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Molecular and evolutionary genetics of invasive bivalves

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MOLECULAR AND EVOLUTIONARY GENETICS OF INVASIVE BIVALVES

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“Look deep into nature, and then you will understand everything better”

Albert Einstein

Dedicated to my family

Author's declaration

The author states that she provides a major contribution to the conceptual design and execution of the work, interpretation of the results and preparation of the manuscript. The following published, submitted articles were prepared under the scope of this dissertation:

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Abstract

Over the last decades, bivalve biological invasions raised great interest within the scientific community, especially in freshwater, estuaries and marine ecosystems that are more vulnerable to this phenomenon. The bivalve bioinvasions provoke significant harmful impacts in the structure and functioning of the invaded ecosystems, disrupting the existent native fauna biodiversity. Moreover, many human infrastructures are also affected by these aquatic invasions, causing significant economic losses in order to control and maintain the sustainability of these human system facilities (*e.g*. water systems need to be frequently unclogged to remove these bivalves). Many researchers have performed local and/or regional ecological and molecular studies to evaluate the environmental impacts caused by invasive bivalves aquatic ecosystems. Since invasive bivalves possess unique morphological and physiological features that contribute to their high potential invasive behavior, it is a complex task to study and evaluate the caused negative impacts in an ecosystem. Yet, remarkable advances in this field of research have been achieved, namely by taking advantage of sensitive molecular genetics methods in order to assess the genetic diversity of invasive bivalves populations.

The main goal of this thesis is to increase the knowledge of three relevant invasive bivalve species, *Corbicula fluminea*, *Dreissena polymorpha* and *Ruditapes philippinarum*, through the use of molecular approaches further supported these species ecologic aspects. The genetic diversity evaluation of these bivalves assists in deciphering the genetic signatures of its natural distribution relatively to possible introductory routes. Herein, new insightful inferences were achieved, which will assist future research studies concerning aquatic invasive bivalve invasions, specifically regarding the ecosystem biodiversity conservation and management guidelines.

This thesis entitled “Molecular and evolutionary genetics of invasive bivalves” consists of three research chapters (**Chapters 2 - 4**) that evaluate the genetic characterization of the three invasive species studied, namely *Corbicula fluminea*, *Dreissena polymorpha* and *Ruditapes philippinarum*. Both *C. fluminea* and *D. polymorpha* are invasive freshwater bivalves, whereas *R. philippinarum* is an invasive marine bivalve.

In **chapter 2**, entitled **“Low genetic diversity and high invasion success of *Corbicula fluminea* (Bivalvia, Corbiculidae) (Müller, 1774) in Portugal”,** it is

described the genetic characterization (using two mitochondrial markers, mtDNA COI, mtDNA CYTb; one nuclear marker 18S rDNA), along with morphometric analyses, and the sperm morphology assay of *C. fluminea.* Specifically, 13 Portuguese ecosystems (Minho, Lima, Tâmega, Tua, Sabor, Douro, Paiva, Mondego, Tejo, Sado, Mira, Guadiana Rivers and Pateira de Fermentelos Lake) were sampled for the presence of this invasive bivalve and genetic analyses performed in a total of 328 specimens. The main findings of this study revealed that the Portuguese *C. fluminea* presents three dominant haplotypes fixed for each marker. In addition, the morphometric analysis demonstrates significant shell dimension differences between the northern and the centre/southern rivers, (with the exception of the Lima River, which resembles more with the centre/southern *C. fluminea* populations), this is mainly attributed to biotic and abiotic factors. Moreover, the presence of biflagellate sperm and the lack of genetic haplotype diversity suggest that *C. fluminea* populations from Portugal belong to the Asian FW5 androgenetic asexual invasive lineage. Furthermore, besides this species natural traits, the low genetic variability detected in these populations is most probably attributed to their asexual reproductive mode which confers *C. fluminea* a high potential invasive behavior. Regarding this non-indigenous species (NIS) introduction route in Europe it is assumed that discharged ballast water from the North and/or South American continent(s) might have been the main dispersal vector. Nevertheless, the direct introduction into Europe from the Asian native genetic pool should not be discarded.

The **chapter 3**, entitled "**Genetic characterization of two invasive sympatric bivalves *Corbicula fluminea* (Müller, 1774) and *Dreissena polymorpha* (Pallas, 1771) in Northern Italy"**, focuses on the genetic assessment of two invasive species, *C. fluminea* and *Dreissena polymorpha* from Italy employing one molecular marker, the mtDNA COI. Two Italian lakes (Lakes Maggiore and Garda) were genetically screened for *C. fluminea*, while the *D. polymorpha* was only sampled in Lake Maggiore. For this study a total of 228 *C. fluminea* and 98 *D. polymorpha* specimens were genetically analyzed employing the mitochondrial COI marker. This research revealed that the Italian *C. fluminea* populations present an identical haplotype diversity pattern as *C. fluminea* from Portugal. Only one main mtDNA COI haplotype was detected, which also belongs to the Asian FW5 androgenetic asexual invasive lineage. Since this Italian NIS present the same genetic pattern as the Portuguese *C. fluminea* populations, it is plausible to infer that the introductory route(s) are also identical as aforementioned (**chapter 2**). Despite of the low genetic diversity detected, it was the first time that these Italian *C. fluminea* populations were genetically analyzed. Regarding the *D. polymorpha,*

two mtDNA COI haplotypes were detected namely, LM1 and LM2 in Lake Maggiore. The LM1 was the main dominant haplotype, whereas the LM2 seems to be a rare haplotype. However, the detected mtDNA COI haplotype comparison with previous studies revealed that the LM1 is also the dominant mtDNA COI haplotype in Lake Garda but LM2 was not detected in Lake Garda. Nevertheless, the LM2 haplotype was previously detected in Germany and thus, seems to corroborate the "German hypothesis" which states that the introduction of Italian *D. polymorpha* population derives from the German genetic pool source but also with other contributions from other European countries. Moreover, from a global distribution perspective, *C. fluminea* presents a worldwide distribution whereas *D. polymorpha* is confined to the North American and European continents. This may be attributed to the different strategic asexual reproductive mode of *C. fluminea* in comparison to the *D. polymorpha* that presents sexual reproduction.

In **chapter 4**, entitled "**Genetic diversity of the invasive bivalve *Ruditapes philippinarum* (Adam and Reeve, 1850) in Portugal"** genetically characterizes 194 *Ruditapes philippinarum* specimens from three locations – Aveiro, Óbidos and Sado – employing two mitochondrial markers (mtDNA COI and mtDNA 16S) and one nuclear marker (18S rDNA). A total of 11 mtDNA COI (COI1-COI11) and five mtDNA 16S (16S1-16S5) were detected. Only one dominant haplotype was detected for the 18S marker and thus, no further analyses were performed. Herein, the three dominant Portuguese *R. philippinarum* mtDNA COI haplotypes (COI3, COI5 and COI6) were detected and are present in most of the European populations, thus suggests the occurrence of multiple introductions from the European genetic pool. Interestingly, mtDNA COI haplotypes COI8-COI11 were detected for the first time in Portugal. Moreover, the network and the phylogenetic analysis exhibit similar patterns, indicating the possibility of two distinct mitochondrial COI haplotype groups between the north/centre (group I - COI4 to COI8, COI10 and COI11) and centre/southern populations (group II - COI1 to CO3 and COI9). Furthermore, the phylogenetic analysis corroborates the distinction of these mitochondrial COI haplotype groups and confirms that the Portuguese *R. philippinarum* derived from a mixture of the Chinese and Japanese populations, where this species were imported accidentally to North America and posteriorly its seeds were imported to Europe. From a global perspective, we can infer that the studied Portuguese *R. philippinarum* populations share the same genetic pool as North American and European populations but with Chinese and Japanese ancestry. This explains the low genetic variability of the mitochondrial COI when compared to the Asian native populations. Regarding the mtDNA 16S, the 16S2 is the dominant haplotype in

Portuguese *R. philippinarum* populations, while other three haplotypes (16S1, 16S4 and 16S5) have been described in the Atlantic and/or Adriatic populations. However, two new haplotypes (16S2 and 16S3) were detected in Portugal for the first time and have also been identified in other European countries and in the Asian native range.

Overall, this thesis provides relevant information regarding three species of invasive bivalves – *C. fluminea*, *D. polymorpha* and *R. philippinarum* – namely, the existent haplotype diversity, comparative analysis between the non-native and the native regions and the possible inference of the introduction route(s). The ecological aspects of these invasive species were also discussed in order to achieve a more complete evolutionary scenario. Finally, this work contributes to corroborate previous genetic characterization studies of *C. fluminea*, *D. polymorpha* and *R. philippinarum* populations, provides additional relevant new genetic information that can be employed for future bioinvasions researches and it contributes to establish management guidelines to control these NIS.

Keywords

Corbicula fluminea, *Dreissena polymorpha*, *Ruditapes philippinarum*, Non-indigenous species, Invasive alien species, Hybridization, Marker, Gene, Cytochrome c oxidase subunit I, Cytochrome b, 16S gene, 18S gene, Haplotypes, Phylogeny, Bioinformatic tools.

Resumo

Nas últimas décadas, as invasões biológicas provocadas por bivalves suscitaram um grande interesse pela comunidade científica, especialmente nos ecosistemas de água doce, estuarinos e marinhos que são os mais vulneráveis a este fenómeno. As bioinvasões por bivalves provocam impactos nocivos significativos na estrutura e no funcionamento dos ecossistemas invadidos, afectando a biodiversidade da fauna nativa existente. Além disso, muitas infra-estruturas humanas também são afectadas por essas invasões aquáticas, causando perdas económicas significativas para controlar e manter a sustentabilidade dessas instalações ao nível dos serviços humanos (*e.g.* sistemas de água que necessitam de ser frequentemente desobstruídos para remover esses bivalves). Muitos investigadores realizaram estudos ecológicos e moleculares, locais e regionais para avaliar os impactos ambientais gerados por bivalves invasores nos meios aquáticos. Dado que os bivalves invasores possuem características morfológicas e fisiológicas únicas conferindo-lhes um grande comportamento invasor, estudar e avaliar os seus impactos negativos num ecossistema é uma tarefa complexa. No entanto, avanços notáveis neste campo de pesquisa foram obtidos, nomeadamente aproveitando os métodos da genética molecular para avaliar a diversidade genética nas populações afectadas por bivalves invasores.

Esta tese tem como objectivo principal aumentar o conhecimento de três espécies relevantes de bivalves invasores, *Corbicula fluminea*, *Dreissena polymorpha* e *Ruditapes philippinarum*, através da abordagem de genética molecular sendo suportada pelos aspectos ecológicos das éspecies. A avaliação desta diversidade genética nestes bivalves permite decifrar as assinaturas genéticas relativas à distribuição natural e possíveis rotas introductórias. Neste estudo novas inferências foram obtidas, que irão auxiliar trabalhos futuros sobre invasões aquáticas por bivalves invasores, especificamente sobre a biodiversidade do ecossistema, assim como directrizes de controlo para a sua conservação e gestão.

Esta tese intitulada "Genética molecular e evolutiva de bivalves invasores" consiste em três capítulos (**Capítulos 2 - 4**) que avaliam a caracterização genética de três espécies invasoras estudadas, nomeadamente *Corbicula fluminea*, *Dreissena polymorpha* e *Ruditapes philippinarum*. Ambas as espécies *C. fluminea* e *D. polymorpha* são bivalves invasores de água doce, enquanto qua a espécie *R. philippinarum* é um

bivalve marinho invasor. O **capítulo 2**, intitulado **"Baixa diversidade genética e um elevado sucesso invasor de *Corbicula fluminea* (Bivalvia, Corbiculidae) (Müller, 1774) em Portugal"** descreve a caracterização genética (utilizando dois marcadores mitocondriais, mtDNA COI, mtDNA Cytb; um marcador nuclear 18S rDNA), análises morfométricas e verificação morfológica do esperma de *C. fluminea*. Especificamente, 13 ecossistemas portugueses (Minho, Lima, Tâmega, Tua, Sabor, Douro, Paiva, Mondego, Tejo, Sado, Mira, Guadiana e Pateira de Fermentelos) foram amostrados para a presença deste bivalve invasor e um total de 328 espécimes foram analisados geneticamente. As principais descobertas deste estudo revelaram que as populações Portuguesas de *C. fluminea* apresentam três haplótipos dominantes fixados para cada marcador. A análise morfométrica demonstra diferenças significativas na dimensão da concha entre os rios do norte e centro/sul (com exceção do rio Lima, que se assemelha mais às populações de *C. fluminea* do centro/sul), este facto encontra-se relacionado com factores bióticos e abióticos. A presença de espermatozóides biflagelados e a falta de diversidade genética haplótipica sugerem que as populações de *C. fluminea* de Portugal pertencem à linhagem asiática FW5, sendo esta caracterizada como uma linhagem invasora androgénica asexuada. Adicionalmente, para além das suas características biológicas, a baixa variabilidade genética detectada nestas populações é provavelmente atribuída ao seu modo de reprodução asexuada, conferindo a *C. fluminea* um elevado potencial de comportamento invasor. Em relação a esta rota de introdução desta espécie não indígena (NIS) na Europa, presume-se que as águas de lastro provenientes do continente Norte e/ou Sul-Americano podem ser o vector principal de dispersão. No entanto, não deve ser descartada uma introdução na Europa directamente proveniente da região asiática com a mesma fonte genética.

O **capítulo 3**, intitulado **"Caracterização genética de dois bivalves invasores simpátricos *Corbicula fluminea* (Müller, 1774) e *Dreissena polymorpha* (Pallas, 1771) no norte da Itália"**, concentra-se na avaliação genética de duas espécies invasoras, *C. fluminea* e *Dreissena polymorpha* de Itália, utilizando um marcador molecular, o mtDNA COI. Dois lagos italianos (Lagos Maggiore e Garda) foram geneticamente analizados para as populações de *C. fluminea*, enquanto que a avaliação genética de *D. polymorpha* só foi analizado o Lago Maggiore. Para este estudo, um total de 228 *C. fluminea* e 98 de *D. polymorpha* espécimes foram geneticamente avaliados utilizando o marcador mitocondrial COI. Esta pesquisa revelou que as populações Italianas de *C. fluminea* apresentam um padrão de diversidade haplotípica idêntico ao das populações de *C. fluminea* de Portugal. Apenas um dos

principais haplótipos do mtDNA COI foi detectado, pertencente à linhagem asiática FW5 androgénica assexuada e invasora. Uma vez que estas espécies não-indigeneas italianas apresentam o mesmo padrão genético que as populações Portuguesas de *C. fluminea*, é plausível inferir que a(s) rota(s) introductória (s) também são identicas às acima mencionadas (capítulo 2). Apesar da baixa diversidade genética detectada, foi a primeira vez que as populações Italianas de *C. fluminea* foram geneticamente avaliadas. Em relação à espécie invasora *D. polymorpha*, dois haplótipos do mtDNA COI foram detectados, nomeadamente, LM1 e LM2 no Lago Maggiore. O principal haplótipo dominante foi o LM1, enquanto o LM2 parece ser um haplótipo raro. No entanto, a comparação de haplótipos do mtDNA COI com estudos anteriores revelou que o LM1 também é o haplótipo mtDNA COI dominante no Lago de Garda, mas o LM2 não foi detectado no Lago de Garda. No entanto, este haplótipo foi previamente detectado na Alemanha, portanto parece corroborar a "Hipótese Alemã", que afirma que a introdução das populações Italianas de *D. polymorpha* deriva da fonte genética alemã. Além disso, de uma perspectiva de distribuição global, a *C. fluminea* apresenta uma distribuição mundial, enquanto a *D. polymorpha* encontra-se confinada apenas no continente Norte Americano e Europeu. Este facto poderá ser atribuído à estratégia dos diferentes modos de reprodução entre estas duas espécies, dado que *C. fluminea* apresenta uma reprodução asexuada comparativamente à *D. polymorpha* cuja reprodução é sexuada.

No **capítulo 4**, intitulado **"Diversidade genética do bivalve invasor *Ruditapes philippinarum* (Adam e Reeve, 1850) em Portugal"**, foi avaliado geneticamente 194 exemplares *Ruditapes philippinarum* em três locais – Aveiro, Óbidos and Sado – empregando dois marcadores mitocondriais (mtDNA COI e mtDNA 16S) e um marcador nuclear (18S rDNA). Um total de 11 haplótipos do mtDNA COI (COI1-COI11) e cinco mtDNA 16S (16S1-16S5) foram detectados. Apenas um haplótipo dominante foi detectado para o marcador 18S rDNA e portanto nenhuma outra análise foi realizada. Adicionalmente foram detectados três haplotipos dominantes no mtDNA COI de *R. philippinarum* (COI3, COI5 e COI6) que estão presentes na maioria das populações europeias, o que sugere que ocorreram múltiplas introduções provenientes da mesma fonte genética. Curiosamente, os haplótipos COI8-COI11 do mtDNA COI foram detectados pela primeira vez em Portugal. Ambas as análises da rede de haplótipos e da filogenia exibem padrões semelhantes, indicando a possibilidade de dois grupos de haplótipos distintos, entre as populações norte/centro (grupo I - COI4 to COI8, COI10 and COI11) e centro/sul (grupo II - COI1 to CO3 and COI9) de Portugal. A análise

filogenética corrobora essa distinção entre grupos de haplótipos e confirma que as populações Portuguesas de *R. philippinarum* derivam de uma mistura das populações Chinesas e Japonesas, dado que esta espécie foi acidentalmental introduzida no Norte da América e posteriormente foi intencionalmente introduzida na Europa. De uma perspectiva global, podemos inferir que a população estudada de *R. philippinarum* compartilha a mesma composição genética que as populações da América do Norte e da Europa, mas com ancestralidade Chinesa e Japanesa. Este facto que explica a baixa variabilidade genética detectada no marcado mitocondrial COI comparativamente às populações nativas da Ásia. Relativamente ao mtDNA 16S, o haplótipo dominante nas populações portuguesas de *R. philippinarum* o é 16S2, enquanto os outros três haplótipos (16S1, 16S4 e 16S5) foram descritos nas populações Atlânticas e/ou Adriáticas. No entanto, dois novos haplótipos (16S2 e 16S3) foram detectados pela primeira vez em Portugal mas também foram identificados em outros países europeus e na região nativa da Ásia.

Em geral, esta tese fornece informações genéticas relevantes sobre três espécies de bivalves invasores - *C. fluminea*, *D. polymorpha* e *R. philippinarum* - nomeadamente, a diversidade haplótipica existente, a análise comparativa entre as regiões não-nativas e nativas e permite fazer possíveis inferências sobre a(s) rota(s) de introdução. Os aspectos ecológicos dessas espécies invasoras também foram discutidos para a obtenção de um cenário evolutivo mais completo. Finalmente, este trabalho contribui para corroborar estudos sobre a caracterização genética nas populações de *C. fluminea*, *D. polymorpha* e *R. philippinarum* previamente realizados, providenciando dados genéticos relevantes que podem ser utilizados para futuras pesquisas em bioinvasões e contribuir para o desenvolvimento de directrizes de conservação e gestão para controlar estas éspecies não-indigeneas.

Palavras-chave

Corbicula fluminea, *Dreissena polymorpha*, *Ruditapes philippinarum*, Espécies não indígenas, Espécies alienígenas invasoras, Hibridização, Marcadores, Gene, Citocromo c oxidase subunidade I, Citocromo b, Gene 16S, Gene 18S, Haplótipo, Filogenia, Ferramentas de bioinformática.

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List of Abbreviations

A	Adenosine
AIS	Alien invasive species
ATP	Adenosine triphosphate
BI	Bayesian Inference
bp	Base pairs
C	Cytosine
C. fluminea	*Corbicula fluminea*
C. madagascariensis	*Corbicula madagascariensis*
C/S	Centre/South
COI	Cytochrome c oxidase subunit I
CYTb	Cytochrome b
D. bugensis	*Dreissena bugensis*
D. p. anatolica	*Dreissena polymorpha anatolica*
D. p. gallandi	*Dreissena polymorpha gallandi*
D. p. polymorpha	*Dreissena polymorpha polymorpha*
D. polymorpha	*Dreissena polymorpha*
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
ESS	Estimated Sample size
F_{ST}	Pairwise fixation index
FW1	Invasive lineage FW1
FW17	Invasive lineage FW17
FW4	Invasive lineage FW4
FW5	Invasive lineage FW5
G	Guanine
GTR + I + γ	General Time Reversible + Invariant + Gamma
h	Haplotype
Hd	Haplotype diversity
HKY + I + γ	Hasegawa-Kishino-Yano + Invariant + Gamma

Kb	Kilobase
LED	Linearized evolutionary distances
MgCl2	Magnesium chloride
min	Minutes
ML	Maximum Likelihood
mM	Millimolar
mtDNA	Mitochondrial DNA
N	Number
N	North
N. limosa	*Neocorbicula limosa*
nDNA	Nuclear DNA
NIS	Non-indigeneous species
no.	Acession number
ºC	Celsius
p	*p* value
p.	*polymorpha*
PC1	Principal component analysis
PC2	Principal component X axis
PCA	Principal component analysis
PCR	Polymerase chain reaction
pmol	Picomole
PSRF	Potential Scale Reduction Factor
RNA	Ribonucleic acid
R. decussatus	*Ruditapes decussatus*
R. philippinarum	*Ruditapes philippinarum*
R. variegatus	*Ruditapes variegatus*
s	Seconds
SNP	Single nucleotide polymorphism
sp.	Specie
spp.	Species
T	Thymine
tRNA	Transfer RNA
rRNA	Ribossomal RNA
w/v	Weight/Volume
100x	Microscope amplication

µl	Microlitre
µM	Micro-molar
µm	Micrometre
16S rDNA	16S ribossomal DNA
18S rDNA	18S ribossomal DNA
π	Nucleotide diversity
%	Percent
±	Plus and minus
≥	Greater than or equal to

CHAPTER 1

General Introduction

1.1. The rise of invasive science

Nowadays, five major causes can originate loss of the biodiversity in an ecosystem namely, habitat destruction, pollution, species over-exploitation, climate change, and most important invasive species (Richardson, 2015). Invasive species are generally defined as non-indigenous species (NIS) that have dispersed from their native range and established in novel habitat thus, provoking negative environmental and economic impacts (IUCN, 2017). Even though, few invaded habitats have not been affected by these invasive NIS, as not all invasive species cause devastating impacts in the invaded novel habitat (Richardson, 2015). For thousands of years organisms have been moved from one place to another but these transferences occurred at a slow rate, involving small numbers of individuals and over short distances (Richardson, 2015). Therefore, this did not cause major disturbances in the novel ecosystems native fauna because these species displacement was not a constant practice (Richardson, 2015).

Interestingly, in 1831 and 1836, respected scientists such as Charles Darwin, Alphonse De Candolle, Joseph Hooker and Charles Lyell, had already mentioned invasive species in their writings (Richardson, 2015). In fact, Charles Darwin made important and curious annotations regarding the ecology of introduced plants – *Cynara cardunculus* and *Silybum marianum* – both native to the European range, but were also observed in Argentina, South America (Darwin, 1859; Richardson, 2015). Considering De Candolle previous ideas, Darwin generated the first general hypothesis about invasive species, stating that introduced plants species that were not closely related to the native plants were more prone to be successful (Darwin, 1859). However, in the 19th century these observations were merely viewed as curiosities and were not considered as an arising global biodiversity threat issue (Richardson, 2015). Most probably because in the mid-1990s, invasions were documented sporadically and there was a lack of knowledge from the scientific community in understanding and evaluating this problematic issue (Richardson, 2015). In fact, the widespread distributions of invasive species became more noticeable in the Age of Discovery and with the European colonialism, allied with the technological developments mediated by human activities (Richardson, 2015). Hence, many non-native species have been introduced accidentally or intentionally beyond it native range due to globalization consequences mediated by human activities (Elton, 1958; Ruiz & Carlton, 2003; Cox, 2004; Meyerson & Mooney, 2007). However, the main concern does not only rely in the introduction of invasive species in a new environment, the fundamental focus persists in the arising problematic

impacts from the ecological, biodiversity and economic perspectives (Elton, 1958; Pimentel *et al*., 2001; Sousa *et al*., 2009).

Actually, Charles Elton (1958), also known as the father of invasive science, had already pointed out several hypotheses in order to explain and demonstrate the importance of the increasing biological invasions caused by different NIS (Elton, 1958; Richardson, 2015). Moreover, Elton proposed that the biological invasions ought to employ different study field areas and the collaboration of the scientific community at a large scale, in order to resolve these biological invasion problems. Presently, the invasion science studies now incorporate different study fields specialities – biogeography, epidemiology, human history, ecology, genetic methods, impact assessment, and population modelling – in order to fully comprehend these NIS invasions (Sousa *et al*., 2009; Simberloff *et al*., 2013; Lowry *et al*., 2013; Sharma, 2015; Richardson, 2015).

1.2. The controversy between invasive species and biological invasion phenomenon

In the biological invasions field, there are some controversies regarding the definition of invasive species (Colautti & MacIsaac, 2004), which reflects the lack of knowledge behind concept of how to characterize a biological invasion. Therefore, it is important to properly define and untangle these notions in order to get a clearer picture of what can be considered an “invasive specie” and a “biological invasion”. Several terms are widely employed as synonyms for the term invasive species, such as: alien (Crawley *et al*., 1996), exotic (Green, 1997), non-indigenous (Pimentel *et al*., 2000; Mack *et al*., 2000; Kolar & Lodge, 2001), imported (Williamson & Fitter, 1996), introduced (Lonsdale, 1994), non-native (Davis *et al*., 2000), immigrant (Bazzaz, 1986), colonizer (Williamson, 1996) and naturalized (Richardson, *et al*., 2000). All these terms reveal an inaccurate perception of what is an invasive species which in turn difficults how to determine a true biological invasion and this was not defined by Elton (1958), the “father” of the invasion science. Since then, many definitions of “biological invasion” have been recommended by several researchers (Davis & Thompson, 2000; Richardson *et al*., 2000; Richardson & Pysek, 2004; Colautti & MacIsaac, 2004). However, despite of the made efforts in trying to obtain a consistent definition for the term “biological

invasion" but disagreements regarding this matter still persist. This is partially attributed to the applicability absence of the geographic and impact criteria (Valéry *et al*., 2008).

Some researchers consider that, for a species to be considered an "invasive species", they should trespass a geographic barrier over a mandatory substantial distance and present significant dispersal (Richardson *et al*., 2000; Richardson & Pysek, 2004; Colautti & MacIsaac, 2004; Pysek & Richardson 2006). Thus, implicates that neither indigenous nor native species cannot be considered invasive species in an ecosystem even if their prevalence is dominant (Richardson *et al*., 2000; Richardson & Pysek, 2004; Pysek & Richardson, 2006). Others believe that native species can be considered invasive if they become dominant in a new habitat, thus this general idea does not distinguish between native and non-native species (Thompson *et al*., 1995; Davis & Thompson, 2000). In addition, both native and non-native species may possess similar mechanisms and functionalities in the ecosystem, even if there is a preeminent prevalence of a native species in an ecosystem (Thompson *et al*., 1995; Prach & Pysek, 1999; Meiners, 2007). According to these criteria, then native European species that simply spread out of its native range to other adjacent habitats within Europe would be considered invasive (Feare, 1984; Pascal *et al*., 2006). Considering solemnly the geographical barrier and ecosystem species dominance point of views aforementioned, do not seem adjustable to define "invasive species" and a "biological invasion".

Other researchers consider that it is fundamental to also incorporate ecological principles and propose that for a species to become invasive, a major positive or negative impact should be observed in the invaded ecosystem (Davis & Thompson, 2000, 2002; Inderjit, 2005; Valéry *et al*., 2008). While, other investigators believe that the impact itself should not be considered a key factor for determining the biological invasion definition because it gives rise to additional interpretations and it is strenuous to characterize what is the accepted threshold that one can consider the existence of impact (Richardson *et al*., 2000; Daehler, 2001; Rejmanek *et al*., 2002). In addition, the impacts derived by biological invasions are nature dependent and rely in the invader and the occupier species biological traits. In fact, if both invasive and native species occupy two different habitats in the same proportions they may not present identical impacts in both ecosystems (Valéry *et al*., 2008). Therefore, researchers propose that the impact concentration might rely on the accentuated differences that reside in both native and invasive species (Grime, 1998; Dukes & Mooney, 2004; Strauss *et al*., 2006). Then loss and/or gain of some species traits could affect the ecosystem functioning and diversity (Schulze & Mooney 1993; Chapin *et al*., 2000). Moreover, functional diversity is strongly

correlated with species richness and the biological invasions impacts tend to be higher when the native species number is low (Díaz & Cabido, 2001; Hooper *et al*. 2002; Valéry *et al*., 2008).

According to Valéry (2008) the impact criterion alone depends on different factors but its importance should not be diminished, due to its fundamental role in the in impact research field as well as for management guidelines implementation. Therefore, the aforementioned criterions individually – geographical dispersal, species ecosystem dominance and impact – are also not suitable for defining a biological invasion (Valéry *et al*., 2008).

Nevertheless, there are others factors that influence biological invasions which rely in inter-specific competition and competitive advantage (*e.g*. predation mutualism), which accentuates a species superiority over another despite of having the same food resources (Valéry *et al*., 2008). The NIS competitive advantage seems to be result from two ways: *i)* the different evolutionary history between the NIS species and the native one, also known as the "enemy release hypothesis (Blossey & Notzold, 1995; Keane & Crawley, 2002) and *ii)* posterior ecosystem modification (*e.g.* euthrofication and predation), which affects the competition through the selective forces (Byers, 2002). Moreover, environmental change leads alien species overtime to respond with dominance over native species and control the invade environment (Shea & Chesson, 2002). In other words, after a species introduction, the environmental changes may alter due to the new species competitive advantage (*e.g.*natural traits) and the ability to establishment new interactions (*e.g.* weakening or strengthening) the native species that co-habit the same ecosystems (Valéry *et al*., 2008).

Overall, Valéry (2008) has proposed a definition for "biological invasion", which seems to generally characterize the fundamental principles that occur in all biological invasions. Thus, the proposed definition is as follows: a species should trespasses the natural barriers/obstacles in order to proliferate and possess competitive advantage to rapidly spread and became the successfully dominant population within the invaded ecosystem. Nevertheless, the other aspects such as impact, dispersal mode, and propagule pressure may be countersued as secondary effects, but indeed these factors do have extreme importance for the invasive behavior and distribution in the ecosystem.

1.3. Aquatic invasions

The introduction of invasive species is continuously increasing due to several factors – globalization, climate change, among others – and as concern in the not so far future we may encounter a whole new biota diversity spread among most ecosystems (McKinney & Lockwood, 1999; Sousa *et al*., 2009). Consequently, this increments ecological and economic threats (*e.g*. on agriculture, forestry and fisheries) which may impair the development and sustaintability of industries in different countries (Pimentel *et al*., 2000, 2001). Thus, it is necessary to implement appropriate measures in order to avoid or at least diminuish the negative impacts and to preserve the existent biodiversity in invaded ecosystems (Grosholz, 2002; Korsu *et al*., 2007).

The aquatic ecosystems are being heavily impacted by NIS, which may have a destructive effect provoking biodiversity loss and ecological mal function of the aquatic system itself (Dudgeon, 2000; Byrnes *et al*., 2007). It is widely accepted that human mediated activities are the principle cause that originate these biological invasions namely, recreational activities, ship ballast water, canal constructions, fisheries and tourism (Carlton & Geller, 1993; Cohen *et al*., 1998; Ruiz & Carlton, 2003). In addition, these human mediated activities combined with the invasive species biological traits and if the novel environmental conditions are favorable may tend to increase the potential invasion success (Sousa et al., 2008a; Pigneur *et al*., 2011)

1.4. General causes of aquatic biological invasions

Presently, the scientific community has acquired vast knowledge on what causes invasive species to spread and successfully establish themselves in a novel habitat. Nevertheless, particular attention is being dedicated in comprehending the predisposition of NIS in becoming potential invasive, proliferating in novel habitats and in identifying the characteristics within a novel ecosystem that may favor this phenomenon (Blackburn & Duncan, 2001). The potential biological invasion processes are dependent of the interaction between migration pathways, ecology and evolutionary forces during the invasion period (Facon *et al*., 2006). Thus, different authors have proposed three pertinent plausible hypotheses in attempt to describe these aspects.

Firstly, the migration pathway into a novel habitat usually involves human interaction, mostly due to the invasive species isolation and reduced mobility. Once an

invasive species trespass this restriction and occupies a novel habitat, the lack of historical coevolution with the native species is an advantage to the invader. The invasive species may benefit from the absence of specialized enemies, competition, predation and parasites, also known as “the enemy release” hypothesis (Keane & Crawley, 2002; Shea & Chesson, 2002; Torchin *et al*., 2003). Secondly, environmental changes play a crucial role in biological invasions because abiotic and biotic factors in an ecosystem may provide optimal conditions that may be favorable for the invasive species to spread without requiring new adaptations. For instance, species during the last Pleistocene glaciations confined themselves to southern refugee areas, posteriorly when the climate condition became favorable they expanded northwards in response to the climate change. However, this spread was also facilitated by human actions (Byers, 2002). Thirdly, bioinvasions are also result of evolutionary changes in which the invader genetic changes are attributed to combination of evolutionary forces. Although the propagule pressure has its influence in the process, since both the number of introductions and number of introduced species may constitute a negative impact in the invaded habitat (Lockwood *et al*., 2005; Colautti *et al*., 2006).

1.5. Effects of invasive bivalves in a novel ecosystem

Invasive bivalves have the capability of restructuring and affecting the abiotic conditions of a novel ecosystem (*e.g.* light and nutrient availability, habitat complexity and interference of physical transport) (Sousa *et al*., 2009). Usually, when invasive bivalves becomes dominant in a novel ecosystem, it is highly probable to cause significant impacts because of their tendency to constitute high population densities and thus, affecting the native benthic biofaunal community (Grosholz, 2002; Byrnes *et al*., 2007). These impacts have been detected in ecosystem where invasive bivalves – *C. fluminea* (Sousa *et al*., 2008a), *C. gigas* (Ruesink *et al*., 2005), *D. polymorpha* (Strayer, 1999), among others – are dominant within an ecosystem. These bivalves tend physically to modify the novel environment through shell production, filter feeding and bioturbation (Karatayev *et al*., 2007), thus affecting the preexistent ecosystem structure and functioning (Sousa *et al*., 2009).

It has been proposed that invasive bivalves tend to increase the density, biomass and diversity of other organisms in marine and freshwater habitats, despite of the sediment type (soft or rocky bottoms) (Commito & Rusignuolo, 2000; Sousa *et al*.,

2009). In addition, Sousa (2009) demonstrated that areas with high densities of *C. fluminea* tend to increase the ecosystems species diversity (primarily oligochaetes, freshwater sponges and amphipods), in comparison to low densities areas of *C. fluminea*. This is most likely attributed to the empty shells that accumulate in the ecosystems bottom and provides more niche opportunities for other species (Sousa *et al*., 2009). Even though invasive bivalves may benefit the ecosystem (*e.g*. density, biomass and species richness) by providing positive effects in the other invertebrate community, this does not imply that they will provoke the same effects on other species inhabiting the same ecosystem. Despite of these aforementioned benefits provided by invasive bivalves, it is most common that bivalve invasions tend to decrease and/or lead to the extinction of a certain species (Sousa *et al*., 2009).

1.6. Importance of invasive bivalve shells

Substrate provision

The invasive bivalve shells provide a substrate for the attachment of sessile organisms by increasing the bottom area surface thus, promotes the availability for recruitment and colonization of other organisms (Crooks & Khim, 1999; Gutiérrez *et al*., 2003). Bivalve shells may serve as substrate to epibenthic, sessile organisms such as: algae, sponges, insect larvae, cirripedians, bryozoans, anthozoans, tube-building polychaetes, and other bivalves species (Gutiérrez *et al*., 2003). The outer surface of the shells (*e.g.* of *P. viridis*) are also important for the attachment of other organisms – algae, hydroids, free-living and tubiculous polychaetes, barnacles, amphipods and ascidians (Rajagopal *et al*., 2006) – especially in soft sediments areas where these species are unable to attach the muddy and/or sandy bottoms (Gutiérrez *et al*., 2003).

Refugees from predation

The empty shells of invasive bivalves may also increase substrate complexity furnishing microhabitats inside the shells cavities and in between neighboring shells (Gutiérrez *et al*., 2003). Organisms may use the empty shells microhabitats (Botts *et al*., 1996; Thayer *et al*., 1997; Robinson *et al*., 2007; Sylvester *et al*., 2007; Werner & Rothhaupt, 2007) as refugee areas from predators and other physical or physiological

stress (Stewart *et al*., 1998). For example, several researches have demonstrated that *D. polymorpha* shells provide interstitial protection of invertebrates from fish predation (Stewart *et al*., 1999; Dieterich *et al*., 2004; Beekey *et al*., 2004) and that *C. gigas* shells are often used for *Cancer magister* juvenile protection against adult and fish predation (Fernandez *et al*., 1993). Whereas, the shell aggregation at the surface bottoms may also protect organisms from physical and/or physiological stress such as, wave exposure, currents, temperature, and desiccation (Sousa *et al*., 2009).

Fluid transport

The transportation of fluid in dense bivalve shells mats also affects the water flow, sediment infiltrations and the transport of solutes particles (Dame, 1996; Gutiérrez *et al*., 2003). Whereas, in isolated shells areas, erosion and the accumulation of organic matter is more likely to occur, affecting the distribution of other microorganisms (Pilditch *et al*., 1997). If the shell aggregation density does not exceed a certain threshold then water flow is expected to drift over the shells, with slow velocity and turbulence (Nowell & Jumars, 1984). In this case, it actually increases the transport of particles and solutes to the bottom surface (Fréchette *et al*., 1989; Huettel & Gust, 1992; Crooks & Khim, 1999). In addition, the increase of sedimentation is quite common in invasive bivalve shell accumulation, affecting the distribution and density of other organisms – sea-grass beds, salt marshes, and algal mats – that form the habitat (Sousa *et al*., 2009). Man built water treatment and supply systems are also affected by invasive bivalves (*e.g. Corbicula* spp. and *D. polymorpha*), provoking water flow disruptions which lead to diverse pipeline blockage in North America and incomplete filtration water, which affects the drinking water quality (McMahon, 2000; Aldridge *et al*., 2004; Parsons & Jefferson 2006)

Light availability

Invasive bivalves are filter feeders of phytoplankton and significantly diminish these existent stock communities (Caraco *et al*., 1997; Pace *et al*., 1998; Strayer *et al*., 1999; Thorp & Casper, 2002). The decrease of planktonic communities and other suspended particles leads to the increase of water clarity, which in turn allows light penetration in the water column. This promotes the growth of other organisms, resulting

in ecosystem modification thus, the rooted macrophytes and periphyton are primarily developed followed by the phytoplankton communities. This has been observed and evaluated in invaded ecosystems where filter feeding bivalves, such as: *D. polymorpha* (Karatayev *et al*., 1997), *C. fluminea* (Phelps, 1994), *L. fortunei* (MacIsaac, 1996; Sylvester *et al*., 2007) are successfully established. Despite of the observed negative impacts in ecosystems, invasions by filter feeding bivalves also present some positive effects – such as, *D. polymorpha* has been used for the removal of polluted fractions from the water column and increase of water clarity, and *C. fluminea* to reduce water turbidity in fish ponds – and inclusively have been employed to eliminate pollution from certain water bodies (Reeders & Vaate 1992; Reeders *et al*., 1993; Sousa *et al*., 2009).

Sediment rearrangement

The physical composition of the sediments can also be modified by the invasive bivalves, influencing the ecosystems function and processes (Vaughn & Hakenkamp, 2001). Most bivalves are deposit-filter feeders (*e.g. C. fluminea* and *R. philippinarum*), they explore the sediments via filtration for food ingestion (Dame, 1996; Vaughn & Hakenkamp, 2001). Consequently, they deposit ingested particles as feces or pseudofeces, also known as biodeposition, which alters and increases the local sediments through resuspension and transport properties (Dame, 1996; Sousa *et al*., 2009). The sediment incretion has devastating economic and ecological impacts in man-built water systems because it requires frequent dredging. This technique provokes major impacts in the sediment as well on the ecosystem fauna because it may: *i)* remove other large organism (Unionid bivalves); ii) ressuspend heavy metal particles; *iii)* increase water turbidity; *iv)* substitute bottom grain size; *v)* increase organic matter content; *vi)* alter the bottoms porosity; *vii)* decrease water holding capacity; and *viii)* affect the distribution and survival of other organisms (Aldridge, 2000; Vaughn & Hakenkamp, 2001; Sousa *et al*., 2009).

1.7. Importance of biological invasion research

The study of biological invasion is much more than studying introduced NIS in a novel ecosystem. It is a deeper understanding of a complex relationship scenario

between the invasive species and the novel ecosystem. In fact, it takes into account the interactions employing other complementary study fields in order to achieve a more insightful evaluation, in order to unravel the underlying complexity of the biological invasions study.

In this sense the bioinvasions may benefit from the different integration fields, such as, ecology, evolution, and biogeography. This field study integration allows: *i)* invasion to be observed as it occurs; *ii)* if the invasive species introduction time is known, then both genetic characterization and dispersion means can be assessed; *iii)* ecological and evolutionary processes to be investigated at a temporal and spatial scale when species are introduced in different places; *iv)* valuable information to be retrieved in terms of dispersal limitation and the ecosystem structuring by the species; and *v)* additional knowledge regarding the adaptation, extinction and saturation phenomenon provoked by invasive species (Lee, 2002; Sax *et al*., 2005, 2007).

1.8. Population studies through standard molecular approaches

Over the past years, many ecological reports have been published for a variety of invasive regarding their biological traits, geographic and spatial distribution, positive and negative impacts and the overall ecosystem evaluation. However, solemnly the ecology field does not provide other fundamental aspects about the existing genetic biodiversity and possible introductory routes that give rise to biological invasions. Therefore, the molecular field provides relevant information that complements previous taxonomic and ecological data.

The molecular biology field analyzes the deoxyribonucleic acids (DNA), which encodes all the organism genetic information and is the basis of life in all organisms (Figure 1.1) (Dragomir-Cosmin & Savini, 2011). The DNA confines the genes of a cell organism and is located in two sites namely, in the mitochondrial organelle, thus the DNA is designated as mitochondrial DNA (mtDNA) and in the chromosomes of the cell nucleus, designated as nuclear DNA (nDNA) (Butler, 2010).

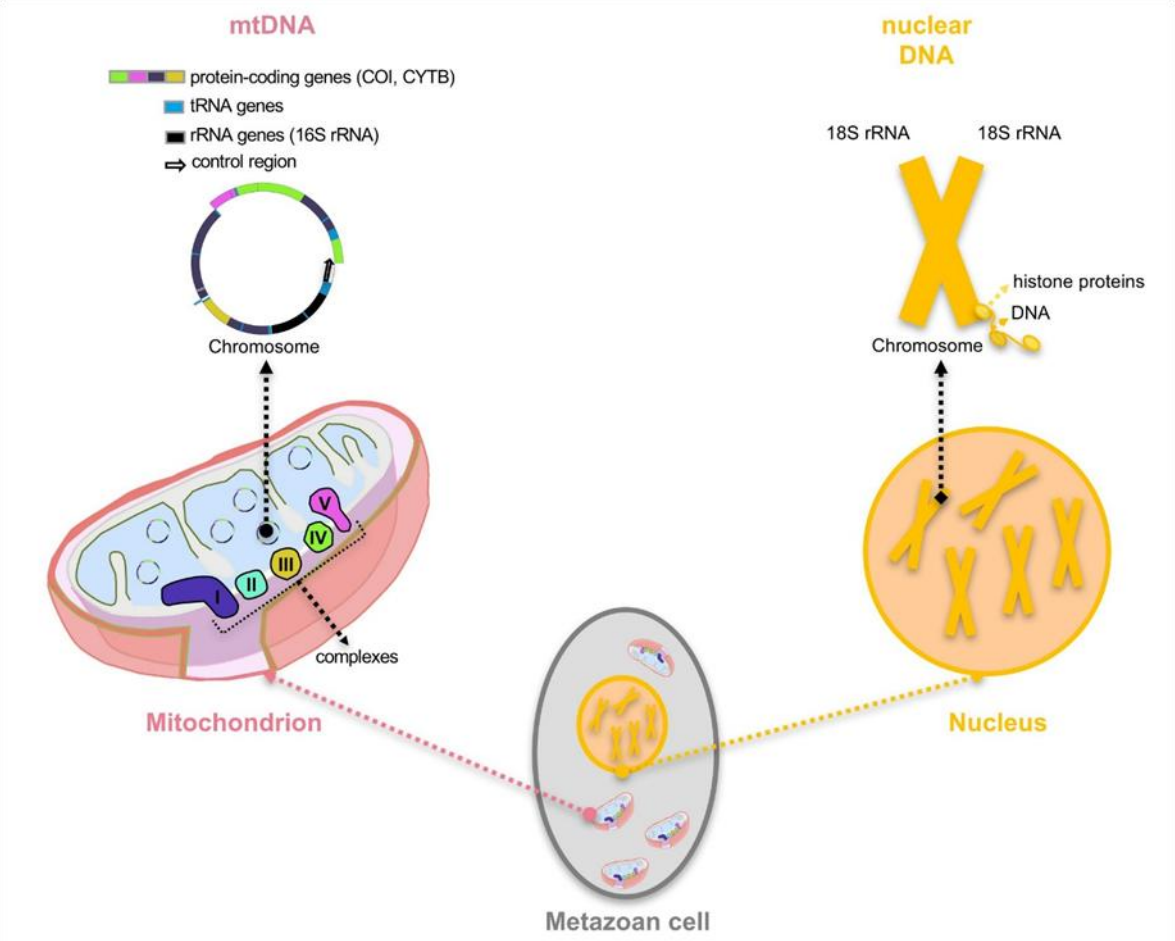


Figure 1.1. Representation of metazoan genomes.Mitochondrial DNA (mtDNA) located inside the cytoplasmic organelle (mitochondrion) and nuclear DNA located inside the chromosomes within the nucleus of a metazoan cell. Named are the genes studied in this work: (i) the mitochondrial protein-coding genes COI – cytochrome c oxidase 1 (encoding for subunits of the complex IV - green), CYTb – cytochrome b (encoding for subunits of the Complex III - yellow), and 16S rRNA – 16S ribosomal RNA; and (ii) the nuclear 18S rRNA – 18S ribosomal RNA (*Adapted from Almeida D, PhD Thesis, 2017*).

Features of the mitochondrial DNA

The mtDNA molecules of most organisms presents special peculiar characteristics, namely: *i)* are relatively smaller than the nuclear DNA molecules (Kolesnikov & Gerasimov, 2012); *ii)* usually are circular molecules and compacted (Osigus *et al*., 2013); *iii)* generally possess 37 genes – 13 protein-coding responsible for encoding the subunits enzyme essential to the functioning of the ATP process synthesis (Burger *et al*., 2003), *iv)* 22 transfer ribonucleic genes (tRNA), which are components of the translation machinery and two ribosomal ribonucleic acids (rRNA) that act as coordinators for protein-coding genes, both tRNA and rRNAs are ribosomal components

that are essential for the translation of the mRNA of protein-coding genes (Burger *et al*., 2003); *v)* do not present introns and other large intergenic regions (Breton et al. 2009; Osigus *et al*., 2013), but present large non-coding regions responsible for the control and transcription and/or replication of mtDNA, known as the control region (Attardi, 1985; Moritz *et al*., 1987; Boore, 1999; Burger *et al*., 2003); *vi)* is excluded of amino acid reparation mechanisms thus, presents higher mutation rates in comparison to the nuclear DNA (Moritz *et al*., 1987); *vii)* both conserved and variable sites are present; *xi)* presents maternal inheritance (with the exceptions of some bivalves where doubly uniparental inheritance (DUI) occurs (Ladoukakis & Zouros, 2001; Passamonti *et al*., 2003); *viii)* englobes specific genetic functions (cell respiration, RNA maturation and protein synthesis import); and *x)* presents a high copy number specific cell and tissue for energy requirements (Boore, 1999; Trinei *et al*., 2006; Kolesnikov & Gerasimov, 2012).

Features of the nuclear DNA

The nuclear DNA presents different features from the mitochondrial DNA (Griffiths *et al*., 2000; Alberts *et al*., 2002). Overall, the nuclear DNA is: *i)* double-stranded and linear; *ii)* presents a higher size (10^8-10^{11} nucleotide per genome) (Saccone *et al*., 2006); *iii)* contains a variable number of genetic information stored in genes, which are distributed throughout a variable number of chromosome pairs (homologous); *iv)* different proteins are associated to the condensing of genetic information and other mechanisms (DNA replication and repair, gene expression,), usually in organism that reproduce sexually, both parental copies are inherited, but in some asexually reproducing bivalves the phenomenon of androgenesis may occur (West, 2003; Hedtke & Hillis, 2010); *v)* presents recombination to assure accurate repair of the existing DNA imprecisions and generates genetic diversity through new gene combinations (West, 2003); *vi)* presents high quantity of repeated non-coding DNA, and *vii)* evolution rate is slower than the mitochondrial protein-coding genes (Vawter & Brown, 1986).

1.8.1. DNA extraction

The scientific market offers several DNA extraction methodologies that can quickly isolate the DNA content (*e.g.* from organisms cells and tissues) in comparison to other classical laborious DNA extraction techniques such as, salting-out (Miller *et al*., 1988) and phenol-chloroform (Sambrook & Russell, 2006). Nowadays, the optimized manual kits and/or robotic automated DNA isolation are mostly used can rapidly allow the extraction of DNA effectively. Basically, the DNA extraction process relies in the presence of chaotrophic salts drive the selective binding of DNA to a silica membrane. This procedure consists essentially of three stages: *i)* the lysis of the material (digestion, protein denaturation and RNA removal), *ii)* DNA binding (through specific binding buffers) and impurities removal through wash buffers, and *iii)* elution of the extracted DNA (Invitrogen, 2017a).

1.8.2. Polymerase Chain Reaction

The polymerase chain reaction, also known as the PCR technique, is an enzymatic process that permits the amplification of a specific DNA target employing a small quantity extracted genomic DNA, independently of the organism tissue (*e.g.* blood, saliva, skin, hair) (Mullis, 1990). Briefly, this methodology demands a mixture of the following components: *i)* DNA template; *ii)* buffer solution; *iii)* $MgCl_2$ solution; *iv)* nucleotides (dNTPs, A = adenine, T = thymine, C = cytosine, and G = guanine) as base blocks to create the final PCR product; *v)* specific primers to the target DNA region to be amplified for the DNA polymerase to extended the amplification; and *vi)* DNA polymerase enzyme - responsible for the nucleotide linkage to the final PCR product, (Garibyan & Avashia, 2013).

Subsequently, the aforementioned PCR mixture is transferred to appropriate PCR tube(s) or plate(s) and placed in a thermal cycler machine which increases or decreases the temperature according to a specific amplification protocol (Weier & Gray, 1988). Basically, three steps occur in the thermal cycler: *i)* the denaturation stage heats up the mixture in order to separate the double stranded DNA; *ii)* the annealing of the primers to the DNA template, in order to bind the primers to the target DNA (note that the annealing temperature is primer specific); and *iii)* extension of the target DNA, the DNA

polymerase inserts the nucleotide for the completion of the DNA strand (Figure 1.2.). The various repetitions of these three steps allow the amplification of the target DNA (Weier & Gray, 1988).

Posterior to the PCR amplification, it is advisable to purify or clean-up the PCR product and ensure the removal of residual components (DNA polymerase, restriction enzymes, dNTPs, primers, buffers and salts), which may contaminate and provoke negative side effects in the later course stages and thus, compromising the obtained data for analysis (Invitrogen, 2017b).

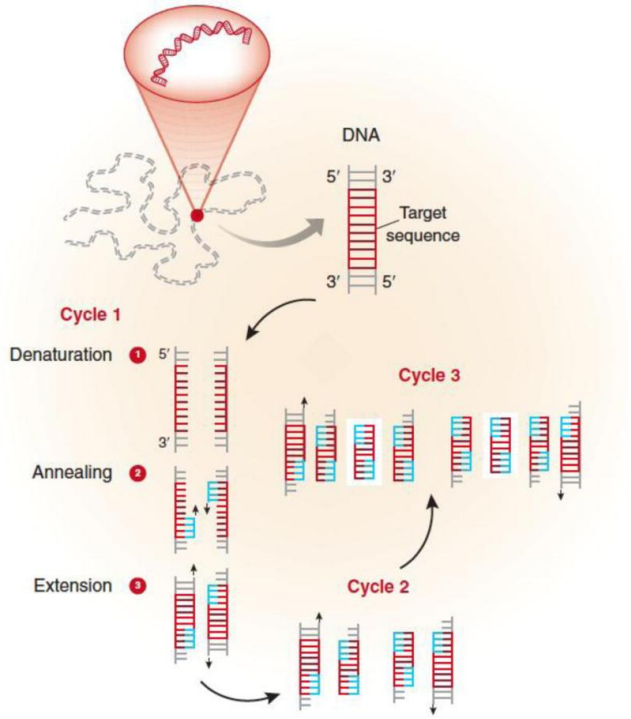


Figure 1.2. Polymerase Chain Reaction stages. The three main PCR stages are represented: denaturation, annealing and extension (Garibyan and Avashia 2013).

Despite of the multiple advantages of the PCR technique (e.g. simplicity and rapidity in obtaining results) some setbacks may occur, such as: *i)* occurrence of sample contamination; *ii)* primer sequence data is necessary in order to perform primer design; *iii)* incorrect detection of target DNA amplification product; *iv)* primers may anneal to other similar non-target DNA areas; and *v)* incorrect insertion of the nucleotide by the DNA polymerase (Smith & Osborn, 2009; Garibyan & Avashia, 2013). However, incorrect primer annealing and nucleotide insertion may occur in very low frequency (Smith & Osborn, 2009). However, the scientific manufactures have considered these

setbacks by producing a variety of robust PCR equipment and proof reading DNA polymerase enzymes in order to avoid these problematic issues (Botes *et al*., 2013; Garibyan & Avashia, 2013).

1.8.3. DNA visualization of post amplification

Posteriorly, the DNA amplification of the PCR products are submitted to an electrophoresis, a procedure which separates the final DNA PCR products according to their molecular size and charge (Figure 1.3.). This procedure employs the usage of: *i)* an electrophoresis machine containing a buffer solution; *ii)* an agarose gel stained with chemical dye for the DNA detection; and *iii)* small amount of the final DNA PCR product. In addition, a specific standardized DNA ladder is also necessary to determine to the final product size.

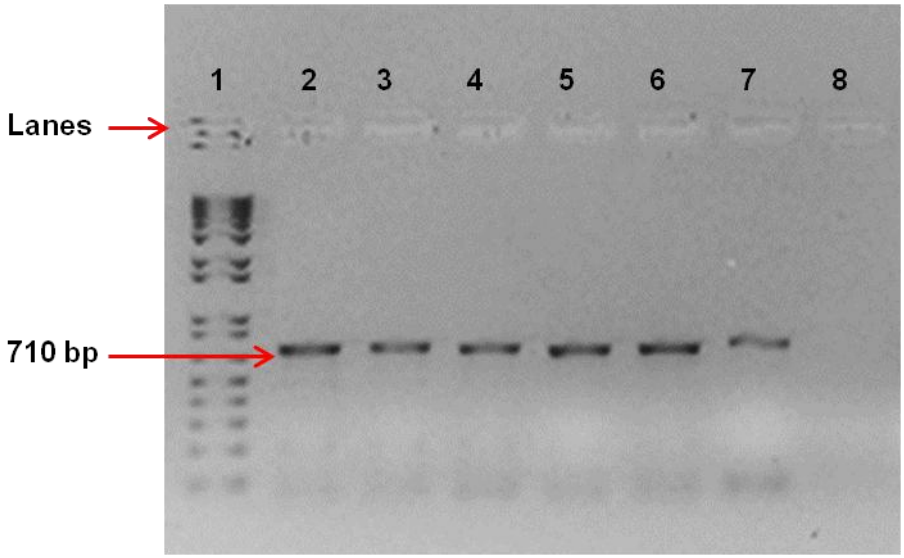


Figure 1.3. Visualization of the final PCR product on agarose gel stained with Redgel dye. First lane is the 1kb ladder, lanes 2-7 are the final PCR products and lane 8 is the negative control.

1.8.4. Sanger-sequencing

The rapid advances in the molecular field led to the accumulation an enormous amount of scientific data which increased a high demand of the research industry to improved DNA sequencing methodology. Currently, the automated DNA fluorescent-

based sequencing analysis is mostly used and it relies in the detection of the target DNA fragment by enzymatically attaching different colored specific fluorophores to the oligonucleotide primer (A, C, T, and G) (Smith et al. 1986). Subsequently, these reactions mixtures are co-electrophoresed in a single polyacrylamide gel tube which detects the fluorescent DNA bands and the sequence information is compiled automatically through computational files (Figure 1.4.) (Smith *et al*., 1986).

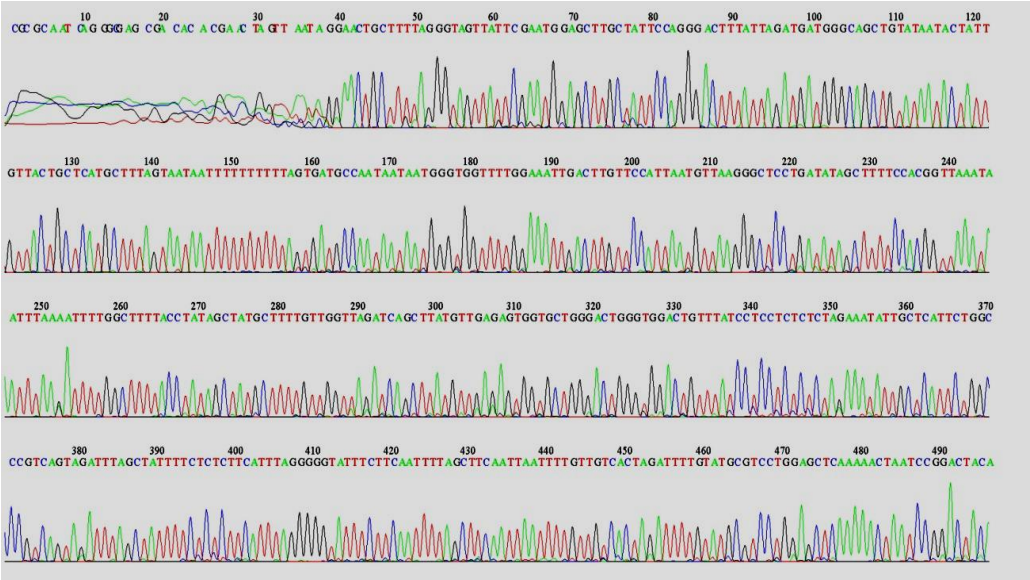


Figure 1.4. **Automated DNA fluorescent-based sequencing output file.**Chromatogram file (from *Macrogen*) showing the nucleotide position, each color (A = green, C = blue, T = red and G = black) corresponds to a specific nucleotide.

This automated DNA fluorescent-based sequencing methodology has eliminated the laborious manual analysis and the associated drawbacks of previous employed classical autoradiography method (*e.g.* avoids the need for re-runs in overlapping gels and the hazardous usage of costly and unstable isotopes). At the present time, there are other alternative DNA sequencing methods that can be employed such as Real Time PCR (RT-PCR) (Eid *et al*., 2009) and Next Generation Sequencing systems (NSG) (which is most adequate for large scale DNA fragments) (Behjati & Tarpey, 2013). Even though, other existing methodologies provide much more additional data, the financial cost is higher and it is also not totally error free. Therefore, it is essential for a researcher to establish the aims of the investigation and select the most adequate DNA sequencing methodology.

1.8.5. Bioinformatics tools for sequence analyses

The human genome sequencing project challenge was the fundamental basis for the "origin and evolution of bioinformatics" as a biological research tool (Sharma, 2015). Following the completion of this enormous research goal – the human genome project – much more biological research was performed, especially in the medical field where the importance of bioinformatics became essential for enhancing the comprehension of the "biological machinery" (Santos *et al*., 2012; Martins & Castilho, 2013; Sharma, 2015).

In the previous decades of the 20th century, molecular biologists generated an enormous amount of data which was an arduous task to handle (Sharma, 2015). The bioinformatics initial objective was to place "some order" in the biological research but with increasing recognition of tool development to analyze generated data in a more efficiently and rapid manner (Edwards *et al*., 2012; Erdogan & Apaydin, 2012; Galindo & Fuente 2012). Another main aim of the bioinformatics relied in tool and technique development in order to allow researchers to fully complete time consuming biological experiments and research to be taken to the further level (Nebel, 2012; Sharma, 2015).

Presently, the bioinformatics area has created the online data availability with efficient manageability, allowing data replacement or allowing modification in a short time period (Sharma, 2015). In the last decades, the bioinformatics fields have made it possible to systematically dissect, assemble, arrange and summarize current biology data through the usage of public databases, such as, Pubmed, NCBI (Bethesda, 2000) and other biological computational softwares (Sharma, 2015). This has been a major contribution to biological research permitting investigators to analyze genes according to specific biologic conditions (Sharma, 2015). Moreover, the generation of on-line public research databases is extremely beneficial for researchers with similar investigation field interests independently of their geographical location (Bethesda, 2000; Sharma, 2015). In addition, bioinformatics has seemed to build a bridge between the biological sciences and other multidisciplinary fields such as, mathematics, computer and statistical science (Sharma, 2015).

Currently, there are several free multitask bioinformatics softwares that allow DNA sequence analysis. The different bioinformatics computational programs enables researchers to further explore the obtained data results in a more rapid and convenient way. For example, some of the common widely used bioinformatics softwares for species genetic characterization studies are: *i)* Mega (Tamura *et al*., 2007) used for the chromatograms visualization and sequence quality check and edition; *ii)* Pubmed and

NCBI online databases employed for sequence comparison (Bethesda, 2000); *iii)* DnaSP and Arlequin used for population genetic statistical analysis (Librado & Rozas, 2009; Excoffier & Lischer, 2010); *iv)* Network analysis v. 5.0.0.0 (Bandelt *et al*., 1999) for determining genetic haplotypes diversity network construction; and *v)* PHYML and MrBayes for the construction phylogenetic tree inferences (Huelsenbeck & Ronquist, 2001; Guindon *et al*., 2010).

Overall, the importance of the existing bioinformatic tool diversity and its biological applicability has been established in all biological research field areas, allowing a rapid analysis of biological raw data in a simpler and faster manner.

Phylogenetic analyses

Posterior to Darwin´s publication "On the origin of species by means of natural selection" (Darwin, 1859), biologists became interested in attempting to reconstruct evolution history of the Earth´s organisms through phylogenetic inferences using paleontological data (Nei & Kumar, 2000). However, paleontological data is usually fragmented and incomplete thus, researchers combined morphological and physiological information in order to unravel the missing information gaps (Nei & Kumar, 2000). However, both morphological and physiological transformations during the organisms evolution is extremely complex and these classical methods did not completely fulfill the organisms evolutionary history and were often controversial (Nei & Kumar, 2000). Presently, due to the advances of molecular techniques moment, the phylogenetic inference allows a greater resolution because it relies in the DNA information data may unravel crucial information about the existing life diversity and how it evolved, especially in the Bivalvia class.

Bivalve investigations are extremely complex and comprehending their ecological role and its genetics is crucial for the preservation and conservation of these species, as well as for the evaluation ecosystems functioning (Sousa *et al*., 2009; Dragomir-Cosmin & Savini, 2011). The Bivalvia class, especially the orders Unionoida, Veneroida, Ostreoida and Mytiloida, is the most targeted for molecular phylogenetic studies, since it includes NIS and some species are of great economic importance for fishery aquaculture purposes (Klinbunga *et al*., 2003; Baker *et al*., 2004; Pie *et al*., 2006; Sousa *et al*., 2009).

The invasive bivalve species phylogenetic molecular approaches have contributed significantly in understanding biological invasions, by determining the existent haplotype

diversity, dispersion and to the possible introductory route(s). In the last years, many researchers have integrated molecular techniques for performing phylogeography inferences in attempt to clarify the invasive bivalve species – *Mytilopsis leucophaeata* (Conrad, 1831) (Therriault *et al*., 2004), *Corbicula fluminea* (Müller, 1774) (Pigneur *et al*., 2014), *Dreissena polymorpha* (Pallas, 1771) (Gelembiuk *et al*., 2006; Quaglia *et al*., 2008) *Crassostrea gigas* (Cardoso et al. 2007), *Ruditapes philippinarum* (Adams & Reeve, 1850) (Chiesa *et al*., 2017) – expansions from their native range and determine the associated introductory vectors of these NIS. In addition, the molecular phylogenetic/phylogeography analyses are also fundamental for bivalve aquaculture and the fishing industries in order to guarantee a rigorous and accurate identification of the bivalve product for consumption (Freire *et al*., 2011; Cordero *et al*., 2017; Chiesa *et al*., 2017). In sum, the molecular phylogenetic approaches are fundamental to evaluate bivalve NIS as well as bivalves with commercial value, since it can untangle many problematic issues (*e.g.* bivalve conservation, ecosystem biodiversity and impact assessment, guarantee bivalve quality, among others). However, integrative approach combining taxonomy, morphological and ecological areas along with the molecular studies should be employed in order to acquire a more enriched knowledge regarding of these bivalve species and ecosystems.

1.9. Invasive bivalves analyzed in this study

The *C. fluminea*, *D. polymorpha* and *R philippinarum* are classified as invasive aquatic bivalves. Both the *C. fluminea* and the *D. polymorpha* are considered the two most aggressive freshwater invasive bivalves. The *R. philippinarum* is a marine invasive bivalve with extreme valuable relevance for the aquaculture/fishing industry but also interferes with the ecosystem biodiversity. Despite of these three species intentional or unintentional introductions in non-native regions they have caused major negative impacts in the ecosystems functioning and/or the existent biodiversity of freshwater aquatic and marine habitats (Sousa *et al*., 2009; IUCN, 2017).

Corbicula fluminea

The *C*. *fluminea* is a NIS from Eastern Asia that has caused major economic and ecological impacts (Figure 1.5). The major ecological impacts are related with trophic

and non-trophic mechanisms in the invaded ecosystem (Sousa *et al*., 2008a; Novais et al., 2015a). This NIS biological traits (McMahon, 2002; Karatayev *et al*., 2007; Sousa *et al*., 2008a) – rapid growth, early sexual maturation, short life span, high fecundity, high filtration rates, broad dispersal capacities (which include natural vectors), ability to inhabit different substrate types, competitive success over native species and interactions with human activities – along with their different reproductive modes (able to reproduce sexually and asexually) and strategies (through androgenesis and mitochondrial egg capture) seems to contribute to their successful invasive behavior (Okamoto & Arimoto, 1986; Komaru *et al*., 1997; Byrne *et al*., 2000; Glaubrecht *et al*., 2003; Pigneur *et al*. 2011, 2012). The *C*. *fluminea* populations may reach great densities and biomass and since it is a filter feeder bivalve, it promotes great changes in existent phytoplankton and zooplankton communities (Sousa *et al*., 2008a; Novais *et al*., 2015a). In addition, the massive presence of shells provokes great changes in water column clarity, bioturbation of sediments, nutrient cycling and substrate colonization by other organisms (Sousa *et al*., 2009; Ilarri *et al*., 2012, 2015). Moreover, *C. fluminea* exhibits a high shell plasticity, mainly due to abiotic (*e.g.* predation) and biotic factors (*e.g*. sediment and water currents) (Sousa *et al*., 2007). Its shell plasticity has originated much confusion in their taxonomic classification status which led to an excessive number of species in the *Corbicula* genus (Britton & Morton, 1982). However, the molecular studies have clarified this issue and supported the existence of fewer species within the *Corbicula* genus (Morton, 1986; Kijviriya *et al*., 1991; Araujo *et al*., 1993; Siripattrawan et al. 2000).

Figure 1.5. *Corbicula fluminea* shell.Outer shell of *C. fluminea* is thick, presents a triangular shape, the coloration may vary from yellow-green or brownish color and possess concentric rings, metric ruler in cm.

C. fluminea populations were first detected outside its native range in 1924 in the North American continent (Counts, 1981; McMahon, 1982) and it is presume this initial introduction in North America served for the purpose of human food source (Britton & Morton, 1979). Subsequently, it spread throughout the North American continent, South America by the 1970s and in the early 1980s was detected in Europe (Mouthon, 1981). Currently, this NIS presents a widespread geographic distribution in Europe, North and South America, and more recently in North Africa freshwater ecosystems (Counts, 1981; Mouthon, 1981; McMahon, 1982; Ituarte, 1994; Clavero *et al*., 2012). This spread can be attributed to different dispersal vectors (*e.g.* commercial transportation through ballast water and other human recreational activities) as well as secondary vectors (*e.g*. birds and mammals) (McMahon, 1982, 2002; Karatayev *et al*., 2007; Belz *et al*., 2012).

Dreissena polymorpha

The NIS *D. polymorpha* is native to Ponto-Caspian region (Figure 1.6) (Mordukhai-Boltovskoi, 1960; Starobogatov & Andreeva, 1994). This species is also responsible for great ecological and economic impacts in the invaded freshwater ecosystems (Pimentel *et al*., 2000; Darrigran, 2002; McMahon, 2002; Karatayev *et al*., 2005). Their physiological traits – rapid growth, early sexual maturation, short life span, high fecundity, high filtration rates, broad dispersal capacities, ability to inhabit different substrate types, competing success over native species and interactions with human activities – contribute to their invasive character, thus increasing their competing success over native species. In addition, *D. Polymorpha* presents the capacity of rapid re-establishment even after catastrophic declines (Cohen *et al*., 1998; McMahon, 2002; Kolar & Lodge, 2002; Karatayev et al. 2007; Belz et al. 2012). Despite of the *D. polymorpha* strict sexual reproduction mode it presents an extremely high invasive potential behaviour.

Figure 1.6. *Dreissena polymorpha* shell.Outer shell of *D. polymorpha* is thin, presents a "D" form shape, color variation but usually are cream colored with a dark brown zig-zag or zebra like pattern, metric ruler in cm.

This NIS dispersal across Eastern and Western Europe freshwater ecosystems occurred in the 1700s - early 1800s, mainly through the water canal systems, built to connect the water bodies for commerce trading (Andrusov, 1897; Zhadin, 1946; Kinzelbach, 1992; Starobogatov & Andreeva, 1994). Posteriorly, the intercontinental introduction in the North American continent was via transoceanic water ballast from commercial transportation (Hebert *et al*., 1989; Claxton *et al*., 1998).

Regarding *D. polymorpha* invasive range, it appears that this species presents a more confined distribution in comparison to *C. fluminea* and it is predominately restricted to the European and North American continent (Kinzelbach, 1992; Bij de Vaate *et al*., 2002; DAISE, 2008). Perhaps, this may be associated to the strict sexual reproduction mode which may be limiting this species spread. However, further studies are necessary to confirm this hypothesis.

Ruditapes philippinarum

The bivalve *R. phillipinarum* (Adams and Reeve, 1850), is a marine successful NIS native to the Indo-pacific native range (Figure 1.7) (Ponurovsky & Yakovlev, 1992) and presents negative ecological impacts such as, displacement of native species due to the continuous increase of these populations (Cohen & Carlton, 1995; Breber, 2002; Juanes *et al*., 2012; Bidegain & Juanes, 2013; Bendell, 2014). In addition, introgressive hybridization events have been detected between *R. decussatus* and *R. phillipinarum*,

which may lead to a significant decrease or even species extinction of the native *R. decussatus* species (Kitada *et al*., 2013; Habtemariam *et al*., 2015).

Figure 1.7. *Ruditapes philippinarum* shell.Outer shell of *R. philippinarum* are thick, with round-triangular shape of presents color variation cream-colored to dark brown, with streaks and defined grid lines, metric ruler in cm.

The *R. phillipinarum* physiological traits – high fecundity, long larval phase, broad salinity and temperature tolerance – (like *C. fluminea* and *D. polymorpha*) seem to confers its successful establishment and proliferation in novel ecosystems (Breber, 2002). In addition, *R. phillipinarum* contributes to the high sustainability of the aquaculture/fishing industry, since it is a highly appreciated shellfish food source (Breber, 2002). Interestingly, the majority of the *R. phillipinarum* production still comes from China (native coastal regions) and followed by Europe (Guo *et al*., 1999; Bald *et al*., 2009).

1.10. Gene sequences analyzed in this study

The success of molecular genetics in population studies relies in the researchers workflow aim and in the application of adequate genetic markers (Sunnucks, 2000). Single locus genetic markers are commonly employed in genetic population studies because: *i)* most of these genetic studies employ one marker and permits comparisons with previous studies; *ii)* single locus are most efficient for evolutionary, population and conservation research; *iii)* they allow the determination gene frequencies, gene flow,

population history and genetic diversity, and *iv)* provide information of the phylogeography, speciation and phylogenetic reconstruction (Davies *et al*., 1999; Taylor *et al*., 2007). It has been proposed that the mitochondrial and nuclear DNA (mtDNA and nDNA) markers are very efficient for the purposes of intraspecific phylogeography and population history and biodiversity assessment (Burke, 1988; Avise, 1994; Simon *et al*., 1994; Templeton, 1998). These molecular assays are important to unravel current and the historical repertoires, such as, haplotype frequency, demographic trends and other informative sequence data (Avise, 1994; Hillis, 1996; Templeton, 1998).

Taken into consideration, in this research both mitochondrial and nuclear DNA markers were employed – COI, CYTb, 16S and 18S – to avoid random biological sampling that present historical pattern differences and to detect discordant biological characters (Sunnucks, 2000). In addition, the haplotype comparison between mtDNA and nDNA markers also allows in the identification of hybrid individuals, stochastic effects on variants in polymorphic ancestral taxa (Sunnucks, 2000). Nevertheless, it is noteworthy to mention that the mtDNA presents a higher conservation rate, which permits these markers to infer demographic scenarios with a higher precision.

1.11. Thesis outline

The genetic characterization of three invasive species *C.fluminea*, *D. polymorpha* and *R. philippinarum* is the main focus of the present thesis. This was accomplished employing a molecular approach using DNA sequences (mitochondrial and nuclear DNA) extracted from the studied samples. The different population genetic methodologies and phylogeographical inferences were performed in order to achieve insightful information regarding these three invasive bivalves genetic diversity, origin and introduction route(s).

This thesis is composed by a general introduction (**chapter 1**), three journal chapters (**chapters 2 - 4**), a general discussion (**chapter 5**), concluding remarks and future perspectives (**chapter 6**). The methodologies used in this thesis are described in the material and methods of each chapter section (**chapters 2 - 4**).

The three foremost experimental contributions of this thesis are divided into three sections:

- **Chapter 2** – consists in the complete assessment of genetic variability of *C. fluminea* (Phylum Mollusca, Bivalvia) in 13 ecosystems of Portugal (Minho, Lima,

Tâmega, Tua, Sabor, Douro, Paiva, Mondego, Tejo, Sado, Mira, Guadiana e Pateira de Fermentelos). Despite of the numerous ecological researches in Portuguese *C. fluminea* populations, there was a scarceness of full genetic characterization. Here, this was accomplished by employing both mitochondrial DNA markers (COI, CYTb) and nuclear DNA (18S) markers, morphometric analysis, morphological sperm analysis and through Portuguese sequences comparison with other available worldwide sequences to establish possible introduction sources and routes.

• **Chapter 3** – focuses in the genetic characterization of two invasive species from Italy namely, *C. fluminea* and *D. polymorpha*. Herein, the genetic screening of *C. fluminea* was evaluated for the first time in the two major Italian Lakes (Maggiore and Garda). While, genetic evaluation of *D. polymorpha* population was only performed in Lake Maggiore. For both of these populations the mitochondrial COI marker was investigated to gather more knowledge of the existent genetic diversity and determine possible introduction(s) source(s).

• **Chapter 4** – infers the genetic evaluation of Portuguese marine invasive bivalve *R. philippinarum* from three different locations within the national territory (Aveiro, Óbidos and Sado). Hence, two mitochondrial markers (COI and 16S rDNA) and one nuclear marker (18S) were employed to evaluate the genetic composition of these populations in order to establish their origin through the genetic characterization and enhance future knowledge of the commercial stocks in the Portuguese *R. philippinarum* hatcheries.

CHAPTER 2

Low genetic diversity and high invasion success of *Corbicula fluminea* (Bivalvia, Corbiculidae) (Müller, 1774) in Portugal

Gomes C, Sousa R, Mendes T, Borges R, Vilares P, Vasconcelos V, et al. (2016) **Low Genetic Diversity and High Invasion Success of *Corbicula fluminea* (Bivalvia, Corbiculidae) (Müller, 1774) in Portugal.** PLoS ONE 11(7): e0158108.

2.1 Abstract

The Asian clam, *Corbicula fluminea*, is an invasive alien species (IAS) originally from Asia that has spread worldwide causing major ecological and economic impacts in aquatic ecosystems. Here, we evaluated *C. fluminea* genetic (using COI mtDNA, CYTb mtDNA and 18S rDNA gene markers), morphometric and sperm morphology variation in Portuguese freshwater ecosystems. The COI marker revealed a single haplotype, which belongs to the Asian FW5 invasive lineage, suggesting a common origin for all the 13 Portuguese *C. fluminea* populations analysed. Morphometric analyses showed differences between the populations colonizing the North (with the exception of the Lima River) and the Centre/South ecosystems. The sperm morphology examination revealed the presence of biflagellate sperm, a distinctive character of the invasive androgenetic lineages. The low genetic variability of the Portuguese *C. fluminea* populations and the pattern of sperm morphology have been illuminating for understanding the demographic history of this invasive species. We hypothesize that these populations were derived from a unique introductory event of a *Corbicula fluminea* FW5 invasive androgenic lineage in the Tejo River, which subsequently dispersed to other Portuguese freshwater ecosystems. The *C. fluminea* asexual reproductive mode may have assisted these populations to become highly invasive despite the low genetic diversity.

2.2 Introduction

Biological invasions by bivalve species have become a worldwide problem due to their dispersal capacity and effects on biological diversity, and ecosystems functions and services (Higgins & Zanden, 2010; Douda *et al*., 2012; Sousa *et al*., 2014). The Asian clam *Corbicula fluminea* (Müller, 1774) is nowadays globally distributed and its invasive success is responsible for major ecological and economic impacts (McMahon, 2002; Darrigran, 2002; Ilarri *et al*., 2012). The successful invasive behaviour of *C. fluminea* may be related to their biological traits, namely: (*i*) rapid growth, (*ii*) early sexual maturation, (*iii*) short life span, (*iv*) high fecundity, (*v*) high filtration rates, (*vi*) broad dispersal capacities (which include natural vectors), (*vii*) ability to inhabit different substrate types, (*viii*) competitive success over native species and (*ix*) interactions with human activities (McMahon, 2002; Karatayev *et al*., 2007; Sousa *et al*., 2008a). Major ecological impacts are related with trophic and non-trophic (engineering) mechanisms (Sousa *et al*., 2008a; Novais *et al*., 2015b). As a filter feeder, this species is responsible for great changes in phytoplankton and zooplankton communities (Sousa *et al*., 2008a; Novais *et al*., 2015b). *C. fluminea* populations may reach great densities and biomass being also consumed by higher trophic levels (Sousa *et al*., 2008; Novais *et al*., 2015b). Regarding non-trophic mechanisms major effects are related with engineering activities, which may be responsible for great changes in water clarity, bioturbation of sediments, nutrient cycling and substrate colonization mainly due to the massive presence of shells (Sousa *et al*., 2009; Ilarri *et al*., 2012, 2015; Novais *et al*., 2015a,b).

Even though the ecological and economic impacts caused by *C*. *fluminea* are substantially documented, their taxonomic status is still unclear. Initially, morphology-based taxonomy led to an excessive number of species within the genus *Corbicula* (Britton & Morton, 1982). This was mainly due to the observed high shell plasticity (Kijviriya *et al*., 1991; Tsoi *et al*., 1991), which is attributed to different biotic (e.g. predation) and abiotic (*e.g.* water current, sediment) factors (Sousa *et al*., 2007). However, additional studies relying mostly on genetic analyses – alloenzymes (Hillis & Patton, 1982; Hatsumi *et al*., 1995; Lee & Kim, 1997) and mitochondrial cytochrome c oxidase subunit I DNA sequences (Renard *et al*., 2000; Siripattrawan *et al*., 2000) – proposed the existence of fewer species (Morton, 1986; Kijviriya *et al*., 1991; Araujo et al., 1993).

Interestingly, the genus *Corbicula* presents different reproductive strategies, being able to reproduce sexually (Okamoto & Arimoto, 1986; Glaubrecht *et al*., 2003) and asexually (Komaru *et al*., 1997; Komaru & Konishi, 1999; Byrne *et al*., 2000; Qiu *et al*., 2001; Korniushin, 2004). Previous reports also indicate that a rare form of asexual reproduction known as androgenesis occurs within the genus *Corbicula*. This phenomenon occurs after the self-fertilization process – by an oocyte and a biflagellate sperm, which is a distinctive character of androgenetic lineages of the genus *Corbicula* found in both native and invasive populations (Konishi *et al*., 1998; Byrne *et al*., 2000; Glaubrecht *et al*., 2003; Ishibashi *et al*., 2003; Park & Kim, 2003; Lee *et al*., 2005; Pigneur *et al*., 2012) – where the maternal nuclear DNA is completely removed, while the retained male pronucleus develops into an "all-male" zygote nucleus, thus giving rise to a progeny of paternal clones (Komaru *et al*., 1997; Konishi *et al*., 1998; Komaru & Konishi, 1999; Ishibashi *et al*., 2003). In addition, "egg parasitism" also known as "mitochondrial DNA capture" may occur between the crossing of two different androgenic lineages of the genus *Corbicula*, with the sperm from one lineage being able to fertilize the egg of another lineage (Lee *et al*., 2005; Hedtke *et al*., 2011). The maternal nuclear DNA of the second lineage is generally mostly extruded from the egg, while the paternal nuclear genome continues to develop but the maternal mitochondrial DNA from the second lineage is captured in this process, giving rise to offspring that possess cytoplasmic-nuclear disjunction (Komaru *et al*., 2001, 2006; Park *et al*., 2002; Lee *et al*., 2005; Hedtke *et al*., 2008; Hedtke & Hillis, 2010; Pigneur *et al*., 2011). However, occasionally during this process, part or the entire maternal nuclear DNA is not completely expelled from the egg, giving rise to "nuclear genome capture" whereby the offspring inherits a hybrid genome (Komaru *et al*., 2006; Hedtke *et al*., 2008, 2011; Pigneur *et al*., 2012). Such distinctive reproductive modes seem to benefit *Corbicula* species fitness and may contribute to the invasive success of the four genus *Corbicula* invasive lineages. Three of these, namely FW1 (form B), FW4 (form Rlc) and FW5 (forms A/R) have been reported in the native (Eastern Asia) and in the non-native range (Europe and North America). The fourth, FW17 (form C/S) has been detected outside the native range but not yet in Eastern Asia (Hedtke *et al*., 2008, 2011; Pigneur *et al*., 2011, 2012).

Currently, *C. fluminea* presents a widespread geographic distribution and has invaded ecosystems throughout Europe, North and South America, and more recently North Africa (Counts, 1981; Mouthon, 1981; McMahon, 1982; Ituarte, 1994; Clavero *et al*., 2012). Records indicate that *C. fluminea* was first detected outside its native range

(Eastern Asia) in 1924 in Vancouver Island, British Columbia (Counts, 1981; McMahon, 1982). By the 1970s it had spread throughout North and South America (Ituarte, 1994) and reached Europe at least as early as the 1980s (Mouthon, 1981). Britton & Morton (1979) suggested that *C. fluminea* was firstly introduced in the North American continent as a food source for humans. However, the introductions into Europe and South America are believed to have occurred via ballast water (Karatayev *et al*., 2007). Consequently, *C. fluminea* dispersed within the continents by different dispersal vectors – commercial transportation and other human activities but also by natural vectors such as birds and mammals – which promoted their rapid spread (McMahon, 1982, Karatayev *et al*., 2007; Belz *et al*., 2012). In Portugal, *C. fluminea* was first detected in the Tejo River in 1980 (Mouthon, 1981) and a few years later was reported in the Douro (Nagel, 1989), Minho (Araujo *et al*., 1993), Lima (Sousa *et al*., 2006), Mondego (Ferreira *et al*., 2004) and Guadiana (Chícharo *et al*., 2000) Rivers. The history of introduction and further establishment of the species in the Tâmega, Tua, Sabor and Sado Rivers and Pateira de Fermentelos Lake are unknown.

The main objectives of this study were to assess the genetic variability and the phylogeography of *C. fluminea* Portuguese populations employing molecular, morphometric and morphological sperm analysis. The obtained results were compared with other available worldwide data of *Corbicula* spp. from native and invaded regions applying population genetics and phylogeographical inference methodologies.

2.3 Material and Methods

2.3.1 Study area and sample collection

A total of 328 specimens of *C. fluminea* were randomly collected in 16 different sites belonging to 13 distinct ecosystems: Minho (four different sites, *N* = 100), Lima (two sites, *N* = 40), Tâmega (one site, *N* = 10), Tua (one site, *N* = 8), Sabor (one site, *N* = 10), Douro (one site, *N* = 10), Paiva (one site, *N* = 7), Mondego (one site, *N* = 30), Tejo (one site, *N* = 30), Sado (one site, *N* = 15), Mira (one site, *N* = 30), Guadiana (one site, *N* = 30) Rivers and Pateira de Fermentelos Lake (one site, *N* = 8), using a scoop net or by handpicking (Figure 2.1). Clams were immediately transported to the laboratory where all the soft body parts were isolated and individually stored at -80 ºC prior to DNA extraction.

River/Tributary	Locality	Sampling site	River Length (km)	Basin area (km²)
Minho	Friestas	M1	75	792
	Valença	M2		
	Lanhelas	M3		
	Engenho	M4		
Lima	Lanheses	L1	65	1 145
	Passagem	L2		
Douro	Gramido	GR	322	18 559
Tâmega	Balteiro	TA	140	
Paiva	Várzea	PV	108	
Tua	Abreiro	TU	106	
Sabor	Felgar	SB	112	
Pateira de Fermentelos (Lake)		PF	---	5
Mondego	Ereira	MO	220	6 772
Tejo	Azambuja (Valada)	TJ	275	24 913
Sado	Álcacer do Sal	SA	175	7 628
Mira	Odeleite	MI	130	1 781
Guadiana	Mértola (Azenas do Guadiana)	GU	260	11 541

Figure 2.1. Hydrological data of the studied Portuguese rivers. Fractions Location of the sampled sites and additional information about length and area of the river and lake basins in Portuguese territory (Dill 1993; Teles et al. 2007).

2.3.2 Genomic DNA extraction, PCR gene amplification and sequencing

Total genomic DNA was extracted from 328 samples of *C. fluminea* foot tissue employing the salting-out method (Miller *et al*., 1988). The mitochondrial genes COI (*N* = 328) and CYTb (*N* = 110) and the nuclear gene 18S rDNA (*N* = 110), were amplified in a total volume of 40 µl per reaction containing: 1x PCR buffer, 2.5 mM $MgCl_2$, 250 µM of each dNTP, 0.5 U of DNA Taq polymerase (Bioline, Luckenwalde, Germany), 10 pmol of a specific set of primers– LCO1490 and HC02198 for the COI (Folmer *et al*., 1994), the HOLLAND18S1 and HOLLAND18S2 for the 18S rDNA (Adamkewicz *et al*., 1997) and CBF6 and CBR6 for the CYTb (Yamada *et al*., 2010). The following PCR cycling conditions were used for the amplification of the mtDNA COI gene: 1 min at 94 ºC for initial denaturation, followed by 35 cycles of 1 min at 94 ºC, 30 s at 45 ºC, 1 min at 72 ºC and final extension of 10 min at 72 °C (Folmer *et al*., 1994). The 18S rDNA gene reactions were performed with the following PCR cycling parameters: 5 min at 95 ºC for initial denaturation, followed by 25 cycles of 4 s at 94 ºC, 2 min at 50 ºC, 1 min at 72 ºC

and final extension of 8 min at 72 °C (Adamkewicz *et al*., 1997). The CYTb amplifications were performed with the following PCR cycling conditions: 2 min at 94 ºC for initial denaturation, followed by 35 cycles of 3 min at 94 ºC, 45 s at 54 ºC, 2 min at 72 ºC and final extension of 5 min at 72 °C (Yamada *et al*., 2010). All PCR products were purified using Diffinity Rapid Tip (Diffinity Genomics, Inc, West Henrietta, NY) according to the manufacturer's instructions. The final PCR amplifications were confirmed by electrophoresis in a 1.5% w/v agarose gel stained with ethidium bromide (Bio-Rad Laboratories Inc., California, USA) and followed by direct sequencing (Macrogen Amsterdam, Netherlands). The NCBI-BLAST program was employed for sequence identification and comparison (Altschul *et al*., 1997; Sayers *et al*., 2009).

2.3.3 Phylogenetic analysis – mtDNA COI, CYTb and 18S rDNA genes

A total of 93 *Corbicula* spp. sequences of the mtDNA COI gene were retrieved from the NCBI-GenBank (Benson *et al*., 2005; Sayers *et al*., 2009) and *Neocorbicula limosa* was used as an outgroup for phylogenetic and phylogeographic analysis. The mtDNA, 18S rDNA and CYTb sequence alignments were performed employing the default parameters of ClustalW in MEGA 6 software (Larkin *et al*., 2007; Tamura *et al*., 2013). DnaSP 5.10 was used for haplotype inference (Librado & Rozas, 2009). Phylogenetic tree construction employed Bayesian Inference (BI) using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) and Maximum Likelihood (ML) using PhyML 3.0.1 (Guindon *et al*., 2010). Both BI and ML employed the GTR + γ + I nucleotide evolutionary model based on the Akaike information criterion (with 95% confidence interval), using the jModelTest 2.1.1 (Guindon & Gascuel, 2003; Darriba *et al*., 2012). The ML analysis used 1000 bootstrap replicates (Guindon *et al*., 2010). The BI analysis was performed employing 5000000 generations, the trees were sampled every 1000th generation and a total of 25% of the generated trees were discarded. The tree convergence was evaluated in MrBayes by analysing the parameters set values of the Potential Scale Reduction factor (PSRF) and the Estimated Sample Size (ESS). In addition, further visual and numeric convergence was assessed using Tracer v1.6 software (Rambaut *et al*., 2014).

2.3.4 Morphometric analysis

The shells of 275 *C. fluminea* specimens – from the Minho, Lima, Mondego, Sado, Mira, Tejo and Guadiana Rivers – were measured for length, height and width using a digital calliper (±0.2 mm). A Principal Component Analysis (PCA) was carried out using the three morphological measurements and the determination of the PCA components was performed using the correlation matrix in the "princomp" function of the R statistical software (Dray *et al*., 2007).

2.3.5 Sperm morphology

A sample of *Corbicula fluminea* (*N* = 10 from the Douro River) was collected to perform sperm morphology analyses. The sperm was obtained by collecting one drop of the specimens fresh gonadal tissue/fluid (Komaru *et al*., 1998) in a glass slide and optical microscopy at 100x magnification was employed to observe the spermatozoa.

2.4 Results

2.4.1 Mitochondrial DNA – COI gene

The obtained mtDNA COI sequences from all the 328 analysed individuals presented a unique haplotype which was phylogenetically compared with 93 other sequences from worldwide *Corbicula* spp. populations retrieved from GenBank (Altschul *et al*., 1997; Benson *et al*., 2005; Sayers *et al*., 2009). Both BI and ML inferences implemented to reconstruct phylogenetic relationships between haplotypes displayed similar topologies (Figure 2.2).

The phylogenetic analysis demonstrated the existence of two well supported clades: the estuarine and the freshwater (1.0/80 and 0.97/79 support values, respectively). The latter clade splits into five groups – I, II, III, IV and V – which includes *Corbicula* specimens from different geographical ranges within Asia, Europe, North and South America, Africa and Oceania (0.97/96 node support) and *C. madagascariensis*, which is an outgroup of the freshwater *Corbicula* spp. lineages. For phylogenetic analysis purposes the classification "Group I-V" was employed in this study and does not imply the existence of haplotype similarity.

The evaluation of Portuguese freshwater populations – Minho, Lima, Tâmega, Tua, Sabor, Douro, Paiva, Tejo, Sado, Mira, Guadiana Rivers and Pateira de Fermentelos Lake – revealed only one mtDNA COI haplotype (belonging to group IV with 0.90/85 node support), which is identical to previously reported haplotypes from Europe (form R), North America (form A), South America and the FW5 invasive lineage from Asia (Figure 2.2).

Group I (0.97/96 node support) encompasses two invasive lineages, the FW1 (form B) from Asia and North America and the FW4 (form Rlc) present in Asia and Europe, whereas group IV (0.90/85 node support) represents the invasive lineage FW5 (form A/R) from Asia, Europe and North America. Both of these groups include COI haplotypes from both the native and non-native range. Groups II and V (1.0/95 and 0.86/91 node support, respectively) are strictly confined to Eastern Asia. Group III (0.69/55 node support) is the only group that includes most of the genus *Corbicula* haplotypes from the non-Asian range, namely from: Europe, South America, Africa and Oceania (with the exception of Israel which is a Western Asian country), as well as the FW17 (C/S form), detected exclusively in non-native regions, namely Europe, Africa and South America.

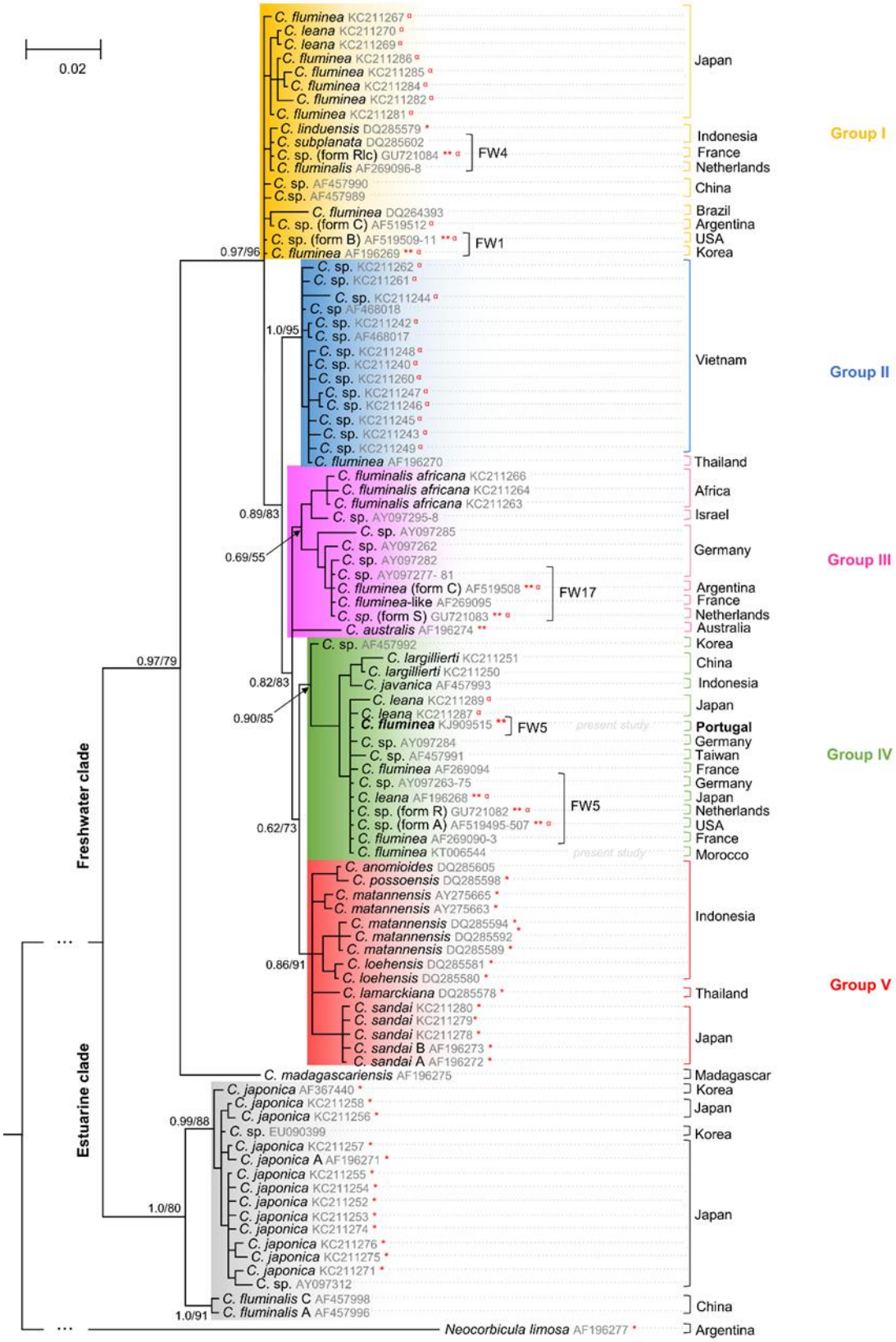


Figure 2.2. **Bayesian phylogenetic tree of the mtDNA COI gene from *Corbicula* genus**. Both Bayesian Inference posterior probabilities (BI) and Maximum Likelihood bootstrap values (ML) are indicated at the nodes.* Indicates the presence of monoflagellate sperm, ** indicates the presence of biflagellate sperm, α represents androgenetic lineages confirmed by cytological studies (Komaru et al. 1998; Glaubrecht et al. 2003; Ishibashi et al. 2003; Pigneur et al. 2014).

CYTb and 18S rDNA genes

A subsample comprising a total of 110 specimens was used to evaluate the genetic variability of the mitochondrial CYTb and the nuclear 18S rDNA genes. The analysis revealed that only two haplotypes – one for the 18S rDNA (accession no. KT878642) and one for CTYb (accession no. KT878643) – were detected in Portuguese *C. fluminea* populations. This finding, suggests that Portuguese *C. fluminea* populations present a low genetic variability for these two markers. Further analyses employing these two markers were not carried out due to insufficient data available in the Genbank database.

Morphometric analysis

The two PCA components explaining most variation (in total 99.4%) were jointly plotted to seek clusters related to the river/region location of each specimen (Figure 2.3). The PC1 and PC2 components suggest the existence of two clusters: north cluster (N), which includes *C. fluminea* specimens from the Minho River; and centre/south cluster (C/S) comprising specimens from Mondego, Tejo, Sado, Mira, Guadiana Rivers. However, the majority (31) of the specimens from the Lima River (N = 40), located in the North of Portugal, grouped within the C/S cluster. Therefore, morphologically they present more similarities with the *C. fluminea* populations from the centre and south.

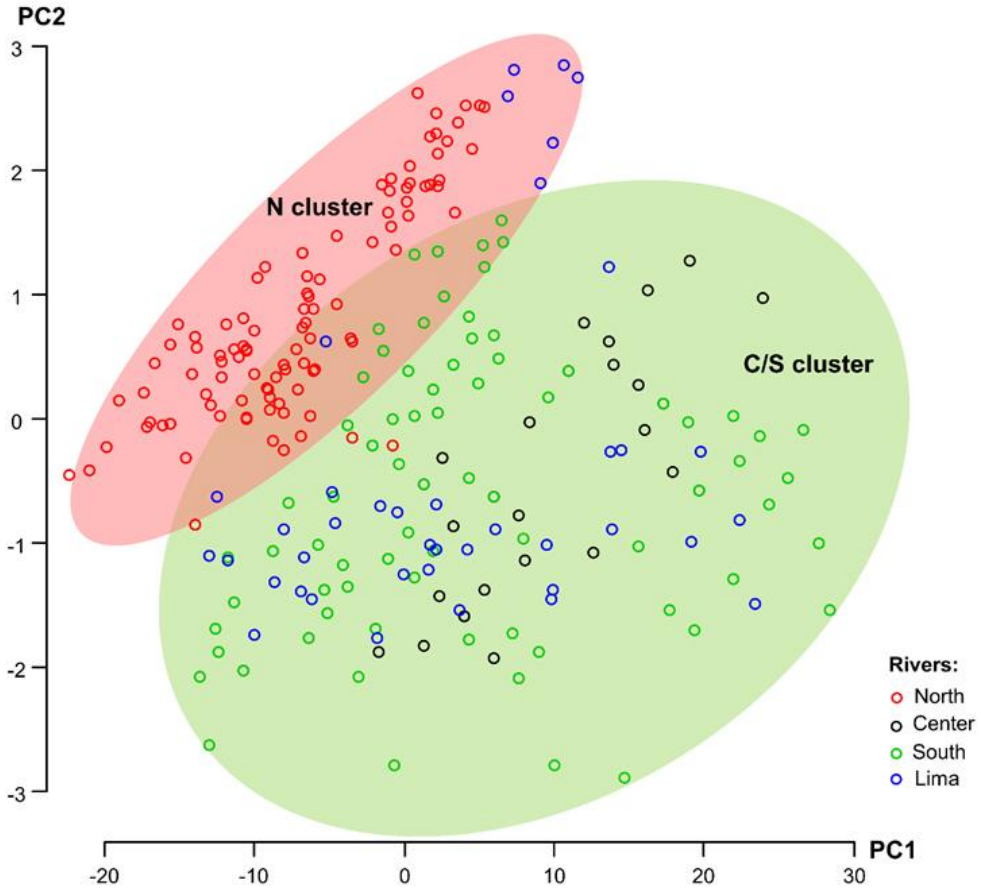


Figure 2.3. **Principal Component Analysis.**The PCA showing the relationship of the PC1 and PC2 components of *Corbicula fluminea* populations from rivers of the North, Centre and South of Portugal, and the Lima River (North Portugal). Each circle represents one specimen corresponding to a specific river. The N cluster in the light red oval circle represents *C. fluminea* populations from the northern rivers and C/S in light green oval represents the centre and southern rivers cluster.

Sperm morphology

The sperm morphology analysis of the subset *C. fluminea* inhabiting the Douro River clearly revealed the presence of biflagellate spermatozoa (Figure 2.4), a distinctive character of androgenetic lineages of the genus *Corbicula*, which are associated with high invasive potential but low genetic variability (Hedtke et al., 2008; Hedtke & Hillis, 2010; Pigneur *et al*., 2011, 2012, 2014).

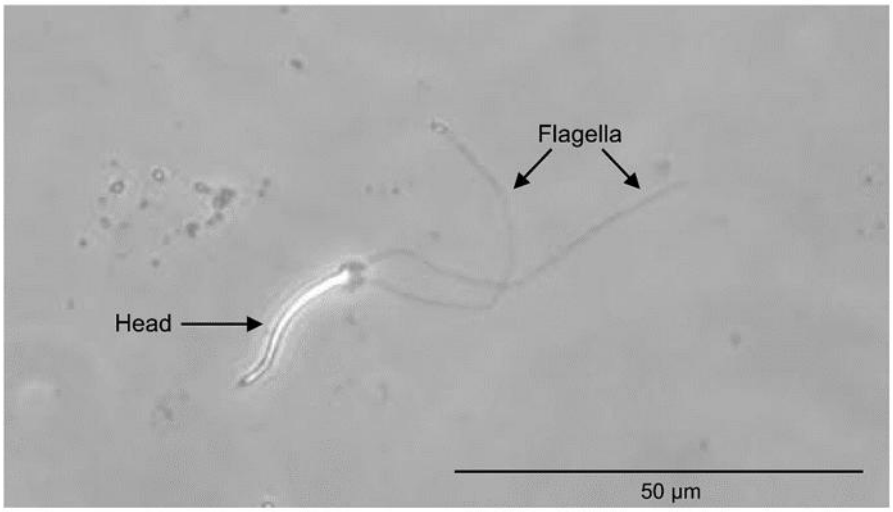


Figure 2.4. Sperm morphology of *Corbicula fluminea* from Portugal.Biflagellate sperm of a specimen of *C. fluminea* from the Douro River (Microscopy photograph acquired by Olympus SZX10 microscope with an integrated Olympus D72 camera).

2.5 Discussion

Genetic and morphological analysis of the Portuguese *Corbicula fluminea* populations

The database sequence comparisons and the phylogenetic inference revealed the existence of a unique mtDNA COI haplotype in the studied Portuguese *C. fluminea* populations belonging to group IV. This haplotype is identical to the European haplotype I (Renard *et al*., 2000), the North American haplotype form A (Siripattrawan *et al*., 2000) and the Asian FW5 haplotype (Park & Kim, 2003). In addition, the FW5 haplotype comprehends the majority of *Corbicula* spp. with biflagellate sperm which is indicative this lineage reproduces through androgenesis, a rare form of asexual reproduction (Hedtke *et al*., 2008; Pigneur *et al*., 2011).

A previous study also reported the existence of this mtDNA COI haplotype in *C. fluminea* populations of the Minho and Lima Rivers (38 out of 41 individuals analysed in six different sampling sites) and three other rare haplotypes (each represented by only one specimen) in the Minho River (Sousa *et al*., 2007). However, in the present study, those rare haplotypes were not observed despite analysis of an eight times larger sample size (*N* = 328). The absence of the rare haplotype most probably resulted from the erosion of the genetic variability due to *C. fluminea* massive mortality events that occur recurrently in the Minho River (Ilarri *et al*., 2011) potentially leading to the loss of these rare alleles by genetic drift after a reduction in the population size (Nei, 1987).

Even though the *C. fluminea* population recovered rapidly from these die-offs and attained its previous biomass and density (Ilarri *et al*., 2011), our data shows the lack of variation of the mtDNA COI in a considerable large number of individuals from the Minho River (*N* = 100).

The genetic analysis of the mtDNA CYTb and 18S rDNA also yielded only one sequence for each marker in the studied *C. fluminea* populations. The mtDNA CYTb haplotype has also been reported in Japanese populations (Yamada *et al*., 2010). The 18S rDNA sequence corresponds to the same previously found in other European and North American populations namely, Spain (Espiñeira *et al*., 2009), the United Kingdom (Taylor *et al*., 2007) and the USA (Giribet & Wheeler 2002; Frischer *et al*., 2002). Therefore, the present study indicates a low genetic variability within the both mitochondrial (COI) and the nuclear marker (18S) in *C. fluminea* populations from the main Portuguese basins. Thus, this seems to be a general pattern in *C. fluminea* Portuguese populations. In addition, the sperm morphology revealed that at least one sample of the Portuguese *C. fluminea* lineage is biflagellate, a distinctive character of the asexual androgenetic lineages (Konishi *et al*., 1998; Byrne *et al*., 2000). Given the lack of genetic variability detected in the studied populations with the employed genetic markers, we hypothesize that the Portuguese *C. fluminea* has derived from an androgenetic invasive asexual lineage with low mitochondrial genetic variability. In fact, some case-studies have reported successful invasions with low or no genetic variation in animals and plants regardless of their reproduction mode – in Africa diverse genotypes of the water flea *Daphnia pulex* have been replaced by a single non-native clone from the American continent (Mergeay *et al*., 2006), introduced populations of invasive Argentine ant *Linepithema humile* in California present a loss of genetic diversity which is associated with reduced intraspecific aggression and form interspecific dominant supercolonies (Tsutsui *et al*., 2000), the Mediterranean bluespotted cornetfish *Fistularia commersonii* exhibits low genetic variability in comparison to the Indo-pacific native range (Golani *et al*., 2007) and the invasive water hyacinth *Eichhornia crassipes* from the Amazon basin presents one main clonal genotype in China (Ren *et al*., 2005; Liu *et al*., 2006). In this case, *C. fluminea* asexual reproductive mode may assist these populations to become highly invasive despite their observed low genetic diversity (Vrijenhoek, 1998; Roman & Darling 2007). However, further studies using other nuclear markers are required to confirm the low genetic variability at the nuclear level. Despite the low genetic variation in the mtDNA (COI and CYTb) and rDNA (18S) in *C. fluminea* populations found in the present study, we hypothesize that an asexual reproduction

strategy might seem to increase their reproductive potential, thus contributing to their high invasive success (Hedtke et al., 2008; Hedtke & Hillis, 2010; Pigneur *et al*., 2011, 2012, 2014). Therefore, considering the invasive history of *C. fluminea* in Portugal, we hypothesize that the low genetic variability found in populations from main rivers all over the country, is a result of the introduction of an asexual lineage with a reduced genetic pool in Tagus River – where this species was first reported – that rapidly spread to other Portuguese freshwater ecosystems. This spread may have occurred through different dispersal mechanisms that may include human activities or natural dispersion by birds, mammals and fish as previously reported (Prezant & Chalermwat, 1984; McMahon, 2002; Karatayev *et al*., 2007). While the Portuguese *C. fluminea* populations exhibited low genetic variability, morphological differences have been detected in the present study (Figure 2.3). Two morphotypes were observed, one corresponding to *C. fluminea* populations from the northern rivers and the other, corresponding to populations from centre/southern rivers. The exception is the population of the Lima River – located in the north of Portugal – that is morphologically more similar to centre/south populations than to other northern populations. The observed morphological differences may be attributed to biotic or abiotic factors that influence shell morphology that may include avoidance of predation and parasitism, different current flow conditions, type of substratum, conductivity and calcium availability, among other factors (Stanley, 1983; Gardner & Skibinski, 1991; Willis & Skibinski, 1992; Norberg & Tedengren, 1995; Baker *et al*., 2003). The morphometric analysis of the studied *C. fluminea* populations may provide some ecologic insight, but further studies employing a hierarchal experimental design – composed of a robust *C. fluminea* sampling, evaluation of densities and the evaluation of both biotic and abiotic factors in these ecosystems – would be necessary to acquire a deeper ecological knowledge of the spatial variability of this species.

Global haplotype diversity and distribution of the genus *Corbicula*

Most of the groups resolved in the phylogenetic inference present polytomies (Figure 2.2), indicating that *Corbicula* spp. dispersal occurred in a short temporal scale. From a global perspective, we can observe that the native range presents higher *Corbicula* spp. haplotype diversity – 40 out of 47 haplotypes – in comparison to the invaded regions (Figure 2.5). However, the confinement or the absence from the species' native range of some *Corbicula* spp. (groups II, V and III, respectively) still remains to be explained (Pigneur *et al*., 2014). Perhaps physiological and environmental

constraints and/or *Corbicula* spp. habitats that are less subjected to human mediated activities may in fact be inhibiting the spread of these haplotypes that derive from well-established populations (McMahon, 2002). Further studies should be performed to address these questions.

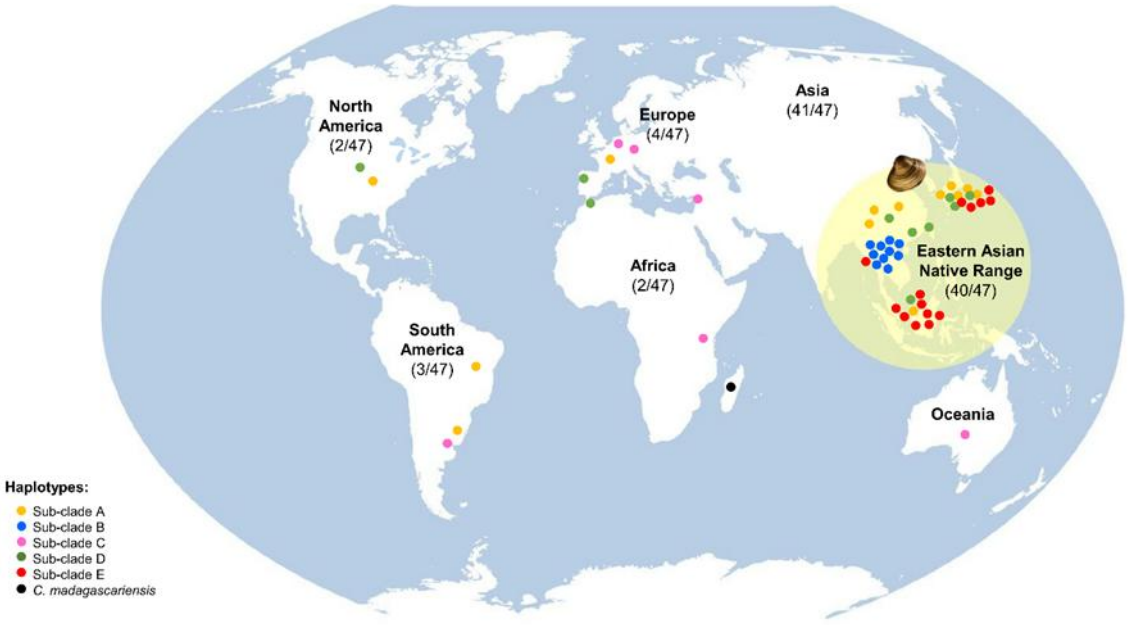


Figure 2.5. Worldwide map representing the *Corbicula* spp. distribution from the native and the non-native range.N indicates the number of haplotypes. Exclusively from the native-range two groups II and V (N = 10 haplotypes and N = 14 haplotypes, respectively). Group I is represented by 9 haplotypes and group IV by 7. The non-native range group I represents 4 haplotypes (North America, N = 1; South America, N = 2 and Europe N = 1). Group III englobes a total of 6 haplotypes; (Africa, N = 1; South America, N = 1; Europe N = 3 and Oceania, N = 1). Group IV presents a total of 3 haplotypes (North America N = 1, Europe N = 1 and Africa N = 1).

2.4.1 The dispersal trajectory of the *Corbicula* spp. invasive lineages

It is generally accepted that the *Corbicula* spp. invasion in Europe was exclusively by water ballast transport from America (Kinzelbach, 1991). However, recent genetic studies are not able to confirm whether the primary introduction of the invasive lineage FW5 (form A/R) in Europe was via North and/or South America (Pigneur *et al*., 2014). However, we cannot exclude the hypothesis that the *Corbicula* spp. may have also been introduced into the European continent directly from the Asian populations (Figure 2.6). The FW1 (form B) and the FW4 (form Rlc) invasive lineages (Figures 2.4 and 2.6) both clustered in group I. It has been proposed that both lineages may possess the same mitochondrial ancestor due to the detection of only one nucleotide difference in the mtDNA COI gene (Pigneur *et al*., 2014). Interestingly, the FW17 (form C/S)

invasive lineage has not been detected in the eastern native range *Corbicula* spp. (Figure 2.5 and 2.6). In fact, a recent population genetic study Pigneur (2014) corroborates this result and hypothesizes an introduction of the FW17 invasive lineage from the African continent and subsequent spread to the South America and subsequently into Europe. Nevertheless, inferring introductions routes for the *Corbicula* species is indeed an arduous task, especially when considering the existent taxonomic controversy in this genus. Perhaps an integrative approach employing ecology, morphology and genetic techniques may provide further insights regarding the invasive dispersal trajectory of this IAS.

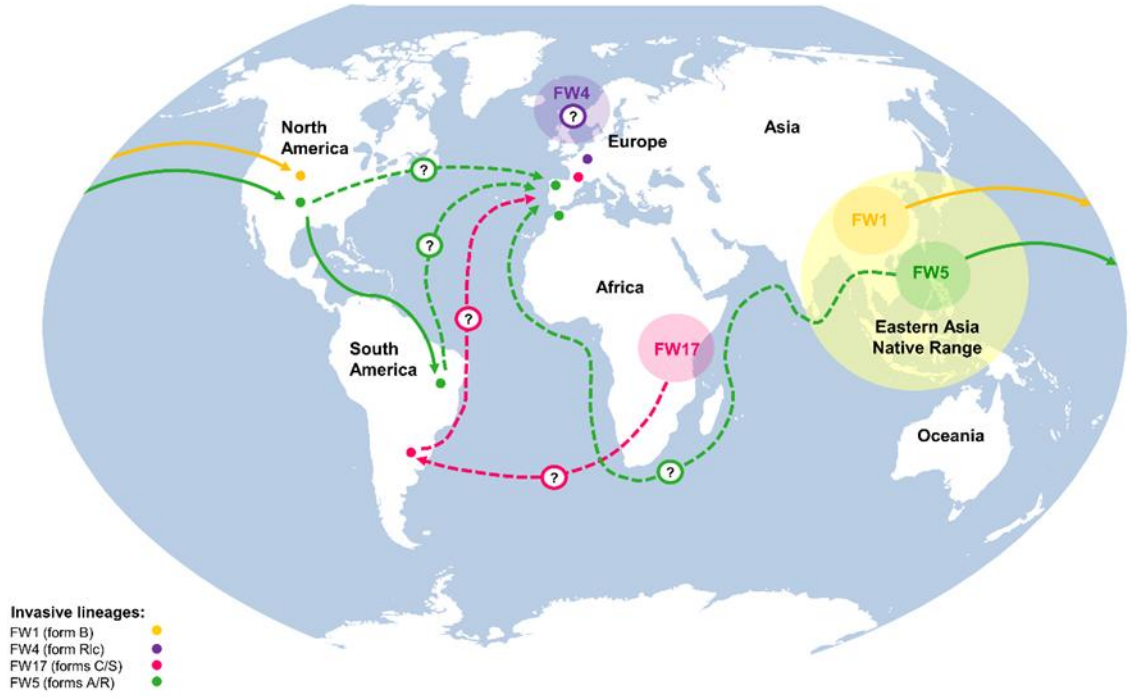


Figure 2.6. Dispersal routes of *Corbicula* spp. invasive lineages.Established *Corbicula* spp. dispersal routes are represented by continuous lines and dash lines correspond to other possible spread routes.

2.5 Conclusion

C. fluminea populations from the main Rivers in Portugal revealed a low genetic variability with the employed genetic markers – COI, CYTb and 18S – despite the large number of individuals detected in the studied ecosystems. The mtDNA COI and the presence of biflagellate sperm indicate that Portuguese *C. fluminea* populations belong to the FW5 androgenetic invasive lineage. Thus, we suggest that a reduced genetic pool was probably recently introduced first in the Tagus River and afterwards spread quickly

to other Portuguese freshwater ecosystems. At the moment is not possible to unambiguously infer neither the *C. fluminea* primary introductory route(s) within Portugal nor the main population source (North America and/or South America or directly from Asia).

CHAPTER 3

Genetic characterization of two invasive sympatric bivalves *Corbicula fluminea* (Müller, 1774) and *Dreissena polymorpha* (Pallas, 1771) in Northern Italy

Gomes C, Mendes T, Borges R, Guarneri I, Marchi I, Vasconcelos V, Guilhermino L, Riccardi N and Antunes A. (2017). **Genetic characterization of two invasive sympatric bivalves *Corbicula fluminea* (Müller, 1774) and *Dreissena polymorpha* (Pallas, 1771) in Northern Italy**. *Submitted to Journal Aquatic Invasions.*

3.1 Abstract

Corbicula fluminea and *Dreissena polymorpha,* are two non-indigenous species (NIS) that have successfully spread from their native range causing prominent ecological and economic impacts in freshwater ecosystems worldwide. Here, we evaluated the COI genetic diversity of *C. fluminea* (Lakes Maggiore and Garda) and *D. polymorpha* (Lake Maggiore) populations from Northern Italy in order to provide insightful information regarding the demographic invasive behaviour of these species. The COI gene analysis revealed one *C. fluminea* haplotype, belonging to the FW5 androgenetic invasive lineage and two haplotypes were found in *D. polymorpha* populations, which have been reported in other European and North American populations. The low genetic variability in the mitochondrial COI marker in both Italian *C. fluminea* and *D. polymorpha* populations from Northern Italy suggests that both of these NIS introductions in Italy originated from a reduced genetic pool, which spread posteriorly to other Italian freshwater ecosystems. Our results contribute for a better understanding of the demographic history of the highly invasive species *C. fluminea* and *D. polymorpha* in Italy.

3.2 Introduction

Corbicula fluminea and *Dreissena polymorpha,* are two non-indigenous species (NIS) native to South Eastern Asia and Ponto-Caspian region, respectively (Mordukhai-Boltovskoi, 1960; Starobogatov & Andreeva, 1994; McMahon, 2000). These NIS have successfully spread from their native range to other worldwide freshwater ecosystems and are liable for great ecological and economic impacts in the invaded regions (Pimentel *et al*., 2000; Darrigran, 2002; McMahon, 2002; Karatayev *et al*., 2005). Globalization has led to the increase of international trade and thus, transoceanic water ballast seems to be the primary vector responsible for the intercontinental spread of these invasive bivalves into other freshwater ecosystems (Kinzelbach, 1991, 1992; Karatayev *et al*., 2007). Both *C. fluminea* and *D. polymorpha* present limited physiological resistance in unstable environments but once established in a new ecosystem, their natural traits – rapid growth, early sexual maturation, short life span, high fecundity, high filtration rates, broad dispersal capacities, ability to inhabit different substrate types, competing success over native species, and interactions with human activities – contribute to their rapid re-establishment after experiencing catastrophic declines and promote their quick spread (Cohen *et al*., 1998; McMahon, 2002; Kolar & Lodge, 2002; Karatayev *et al*., 2007; Sousa *et al*., 2008a; Belz *et al*., 2012).

Interestingly, the *D. polymorpha* reproduction mode is strictly sexual, whereas *C. fluminea* are hermaphrodites and capable of reproducing through a rare form of asexual reproduction known as androgenesis (Okamoto & Arimoto, 1986; Ram *et al*., 1996; Glaubrecht *et al*., 2003). Androgenesis is characterized by the complete removal of the maternal nuclear DNA during the self-fertilization of an oocyte and a biflagellate sperm, thus giving rise to a progeny of only paternal clones (Komaru *et al*., 1998; Komaru & Konishi, 1999; Ishibashi *et al*., 2002, 2003). This reproduction mode seems to favour the invasive potential of *C. fluminea* because under favourable conditions, a single specimen can originate a new population (Hedtke *et al*., 2008, 2011; Hedtke & Hillis, 2010; Pigneur *et al*. 2011, 2014). Despite of the different reproductive modes between *C. fluminea* and *D. polymorpha*, both species present an extremely high invasive potential.

It has been proposed that the primary European introduction of *C. fluminea* (which corresponds to *Corbicula* form A/R) was most probably via ballast water transport from America (Kinzelbach, 1991). Updated studies are not able to confirm whether this primary introduction was via North and/or South America (Pigneur *et al*., 2014) and also

do not discard the hypothesis of a direct European introduction from the Asian range (Gomes *et al*., 2016). Regarding the *D. polymorpha* dispersal, reports suggest that the primary spread was across Eastern and Western Europe through the water canal systems built in the 1700s - early 1800s for commerce purposes (Andrusov, 1897; Zhadin, 1946; Kinzelbach, 1992; Starobogatov & Andreeva 1994) being posteriorly introduced in the North American Continent via transoceanic water ballast (Hebert *et al*., 1989; Claxton *et al*., 1998).

Currently, *C. fluminea* presents a widespread geographic distribution in non-native ranges – European, North American, South American and North African continents (Counts, 1981; Mouthon, 1981; McMahon, 1982; Ituarte, 1994; Clavero *et al*. 2012), whereas *D. polymorpha* invasive range appears to be predominately restricted to the European and North American continent (Figure 3.1) (Kinzelbach, 1992; Bij de Vaate *et al*., 2002; DAISE, 2008). In Italy, *C. fluminea* was first detected in the Po River in the 1990s and subsequently in Lakes Garda and Maggiore in 2002 and 2010, respectively. Reports indicate that *D. polymorpha* was detected in Lake Garda in the late 1960s – early 1970s (Giusti & Oppi 1972). Although no report date exists for *D. Polymorpha* in Lake Maggiore, it is likely that this species was established during the 1990s (Kamburska *et al*., 2013).

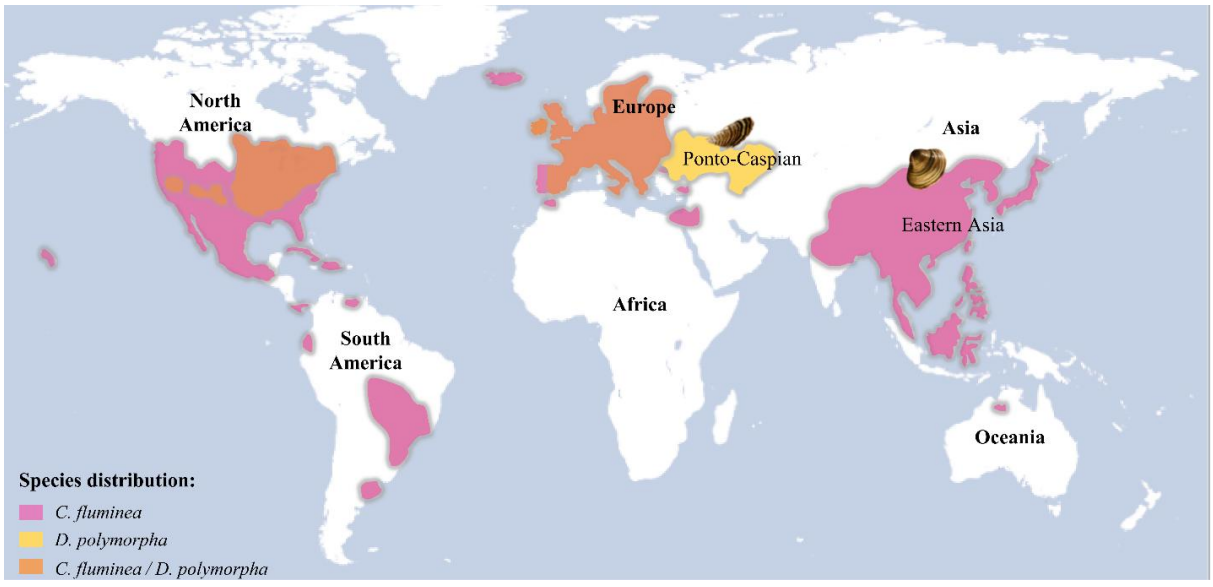


Figure 3.1. Geographical distribution of *C. fluminea* and *D. polymorpha*.The *C. fluminea* (Eastern Asia native range) displays widespread distribution across the continents, while *D. polymorpha* (Ponto-Caspian native region) is confined to the European and North American continents.

Even though many ecological studies have been performed in *C. fluminea* and *D. polymorpha* populations from Northern Italy Lakes, the genetic diversity studies of these invasive species in Italy remain scarce. Thus, we performed for the first time the mitochondrial cytochrome c oxidase (COI) genetic screening of *C. fluminea* populations from the two largest Italian Lakes, Maggiore and Garda, and *D. polymorpha* populations from Lake Maggiore, in order to assess the genetic diversity and to determine the possible introduction population sources and spread of these invasive species in Italy. The obtained COI results were compared with other available worldwide data employing phylogeographical inference methodologies.

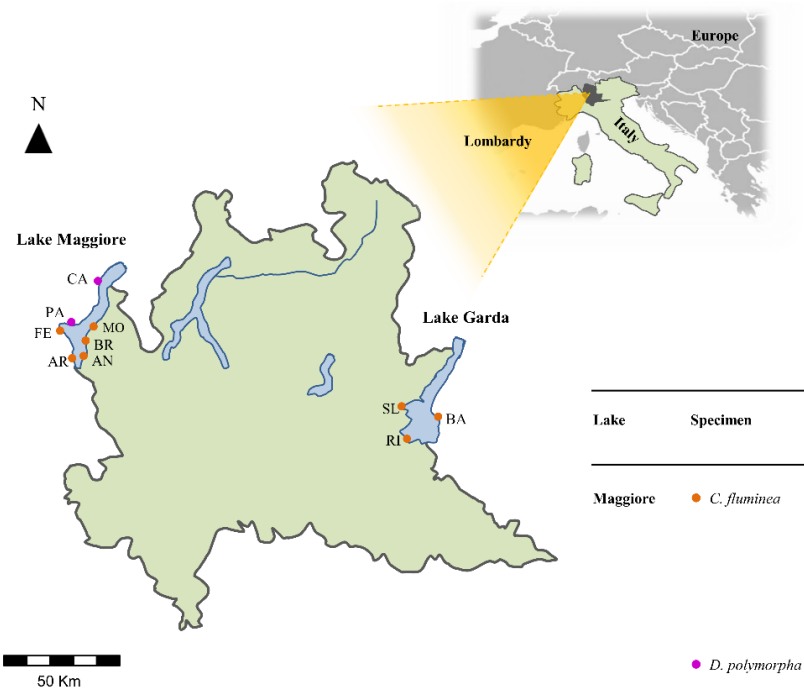
3.3 Materials and Methods

Ethics statement

The study did not involve any kind of endangered or protected species. No specific scientific research permits were required for the sample collection of these highly invasive invertebrates.

Study area and sampling

The sample collection comprised 228 *C. fluminea* from Lakes Maggiore and Garda (5 sites, *N* = 137; 3 sites, *N* = 91, respectively), whereas, 98 *D. polymorpha* samples were collected from Lake Maggiore in two sites (Figure 3.2). The mussels were collected from the lakes using a scoop net and/or by manually handpick. The specimens were transported to the laboratory and the soft body tissues were individually isolated and preserved in absolute ethanol prior to DNA extraction.



Lake	Specimen	Locality	Sampling site	Samples (N)	Basin area (km²)
Maggiore	● *C. fluminea*	Angera	AN	28	212,5 km²
		Arona	AR	27	
		Brebbia	BR	29	
		Feriolo	FE	27	
		Monvalle	MO	26	
	● *D. polymorpha*	Cannobio	CA	42	
		Pallanza	PA	56	
Garda	● *C. fluminea*				368 km²
		Bardolino	BA	40	
		Rivoltella	RI	20	
		Saló	SL	31	

Figure 3.2. Information of sampling area of Lakes Maggiore and Garda. Sampled sites location, number of samples and hydrological data.

Genomic DNA extraction, mtDNA COI amplification and sequencing

Genomic DNA was extracted from the foot or mantle tissue of *C. fluminea* and *D. polymorpha* specimens (*N* = 319) employing the salting-out method (Miller *et al*., 1988). The amplification of a 710 bp fragment of the mitochondrial gene (COI) was performed in a total volume of 40 µl per reaction containing: 1x PCR buffer, 2.5 mM $MgCl_2$, 250 µM of each dNTP, 0.5 U of DNA Taq polymerase (Bioline, Luckenwalde, Germany), 10 pmol of primers set LCO1490 and HCO2198 (Folmer *et al*., 1994). The polymerase chain reaction (PCR) cycling conditions employed were as described by Folmer (1994). The PCR products were purified using NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co., KG Düren, Germany) and visualized on a 1.5% w/v agarose gel stained with GelRed™ (Biotium Inc., Hayward, CA, USA). All final PCR amplifications followed direct sequencing (Macrogen, Amsterdam, Netherlands).

Phylogenetic analysis of the mtDNA COI gene

The NCBI-BLAST algorithm was employed for sequence comparison and nucleotide similarity (Altschul *et al*., 1997; Sayers *et al*., 2009). The phylogenetic analysis were performed by retrieving a total of 95 *Corbicula* spp. and 54 *D. polymorpha* mtDNA COI sequences from GenBank (Benson *et al*., 2005; Sayers *et al*., 2009). Sequence alignments were performed using default parameters of ClustalW in MEGA 6 software (Larkin *et al*., 2007; Tamura *et al*., 2013). The jModelTest 2.1.1 was employed to estimate the best-fit nucleotide substitution models based on the corrected Akaike information criterion (with 95% confidence interval) (Guindon & Gascuel, 2003; Darriba *et al*., 2012). The phylogenetic tree construction of *C. fluminea* employed the GTR + γ +I nucleotide substitution model whereas *D. polymorpha* used the HKY + γ + I criterion. Both phylogenetic trees employed the Maximum Likelihood (ML) method in PhyML 3.0.1 (Guindon et al. 2010), using 1000 bootstraps replicates and Bayesian Inference (BI) in MrBayes 3.1.2, using 5000000 generations (Ronquist & Huelsenbeck, 2003). The BI trees were sampled every 1000th generation and 25% of the generated trees were excluded, tree convergence was evaluated by the Potential Scale Reduction Factor and (PSRF) and the Estimated Sample Size (ESS). The linearized evolutionary distances (LED) which correspond to the sum of the root-to-tip evolutionary distances were calculated using the distRoot (tree,tips) function of the adephylo package of the R statistical software (Pavoine *et al*., 2008; R Core Team, 2017). Statistically significance of the LED between *D. p. anatolica* from Turkey and all the others were tested via the non-parametric Wilcoxon rank sum test for a significance level of 0.05 (Wilcoxon, 1945).

3.4 Results

Corbicula fluminea

The 228 mtDNA COI *C. fluminea* sequences analyzed yielded only one haplotype (accession no. KX231272). This haplotype was compared phylogenetically with other 95 *Corbicula* sequences from worldwide populations (Benson *et al*., 2005; Sayers *et al*., 2009). Similar phylogenetic tree topologies were observed by both BI and the ML inferences, both methods clearly support the separation of the estuarine clade from the freshwater clade (0.99/82 and 0.92/74 support values, respectively) (Supplementary Figure 3.6). The freshwater clade presents five groups – I, II, III, IV and V – englobing

Corbicula species from the native and non-native ranges, namely: Asia, Europe, North and South America, Africa and Oceania and *C. madagascariensis*, which is a sister taxon of the freshwater *Corbicula* spp.

We observed that the detected mtDNA COI haplotype of the Italian *C. fluminea* populations from Lakes Maggiore and Garda – Angera, Arona, Brebbia, Feriolo, Monvalle, Bardolino, Rivoltella and Saló – belongs to the FW5 androgenetic invasive lineage (group IV with 0.96/83 node support) (Figure 3.3). In addition, group IV also shows the presence of this haplotype in other *Corbicula* sp. from both the non-native range – Europe (form R), North America (form A), and South America – and Asian native region.

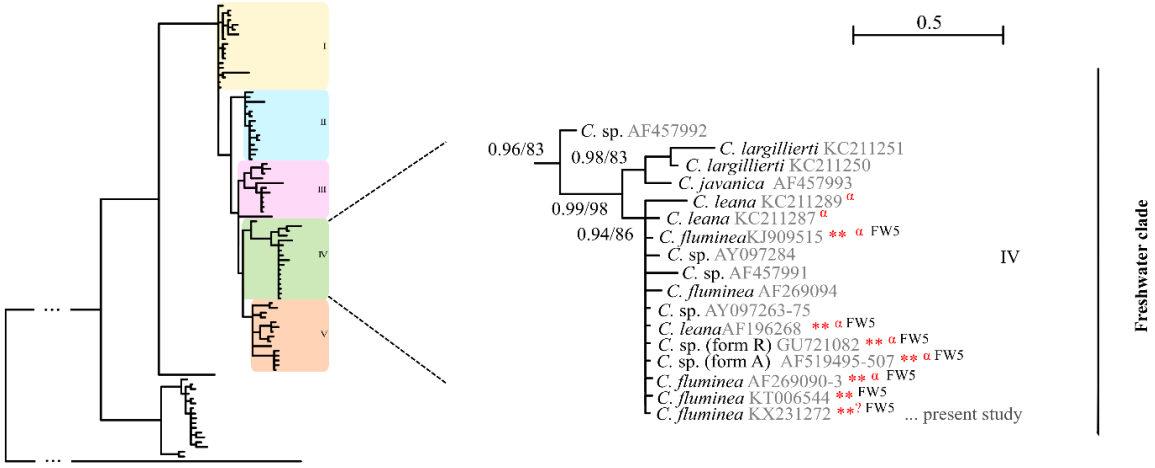


Figure 3.3. Mitochondrial COI phylogenetic group IV subtree of *Corbicula* genus.Both Bayesian Inference posterior probabilities and Maximum Likelihood bootstrap values are indicated at the nodes. * Indicates the presence of monoflagellate sperm, ** indicates the presence of biflagellate sperm, α represents androgenetic lineages confirmed by cytological studies (Komaru et al. 1998; Glaubrecht et al. 2003; Ishibashi et al. 2003; Pigneur et al. 2014).

Dreissena polymorpha

The mtDNA COI genetic evaluation of the 98 *D. polymorpha* specimens studied from Lake Maggiore (Northern Italy), revealed two haplotypes: LM1 and LM2 (accession no. KY884639 and KX231273, respectively). The LM1 haplotype was found in 96 individuals, the LM2 haplotype was only detected in two specimens. Regarding sequence variation, the LM1 haplotype differs from LM2 haplotype by one SNP in the position 354 (C → G). A previous study conducted by Quaglia (2008) reported the LM1 haplotype in the Lake Garda but the LM2 haplotype was detected in Italian population for the first time in the present study. The LM1 and LM2 sequence comparison with other

D. polymorpha sequences (BLAST searches with 100% identity) identify both of these haplotypes in European and North American *D. polymorpha* populations (Benson *et al*., 2005; Sayers *et al*., 2009).

The two haplotype sequences – LM1 and LM2 – were phylogenetically compared with 54 *D. polymorpha* sequences from other populations available in Genbank (Benson *et al*., 2005; Sayers *et al*., 2009). The BI and the ML inferences employed for phylogenetic tree reconstruction displayed similar topologies (Figure 3.4). The phylogenetic analysis clearly reveals the existence of two groups – group I and II. Group I comprises a basal group exclusive of local native *D. p. anatolica* species from Turkey. Group II (0.85/77 node support) includes both the mtDNA COI LM1 and LM2 haplotypes detected in the present study, as well as other haplotypes from *D. polymorpha*, *D. p. polymorpha* and *D. p. gallandi* European and North American populations. In addition, the evolutionary distances (LED) analysis among *D. polymorph*a haplotypes revealed that *D. p. anatolica* species (group I) present significantly lower LEDs (Wilcoxon rank sum test, $p = 2.674 \times 10^{-4}$; Figure 3.5) in comparison with all other *D. polymorpha* species from the Ponto-Caspian native range and non-native ranges across Europe and North America (group II).

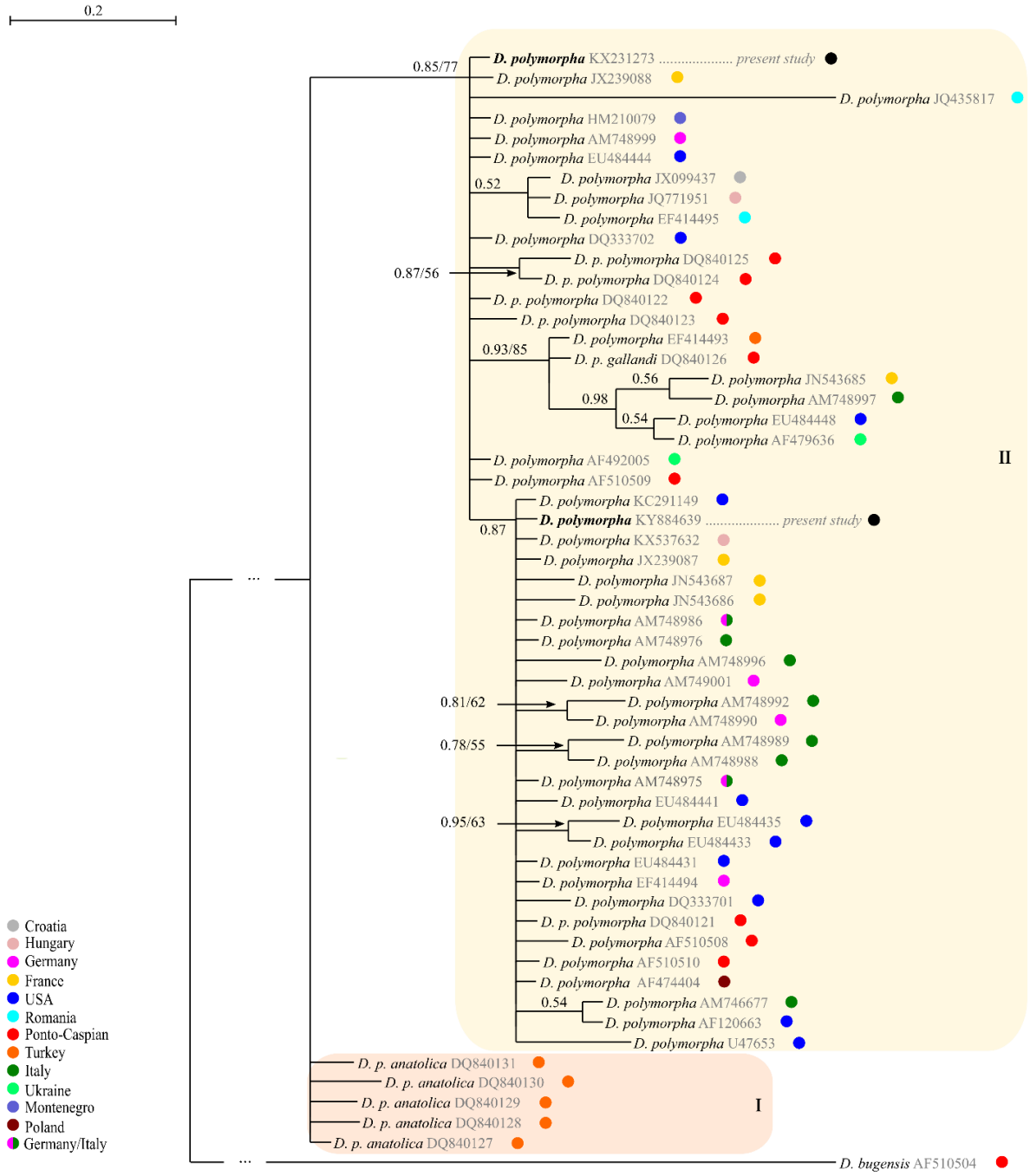


Figure 3.4. Mitochondrial COI phylogenetic phylogenetic of *D. polymorpha*.Both Bayesian Inference posterior probabilities and Maximum Likelihood bootstrap values are indicated at the nodes. The coloured circles indicate the species location.

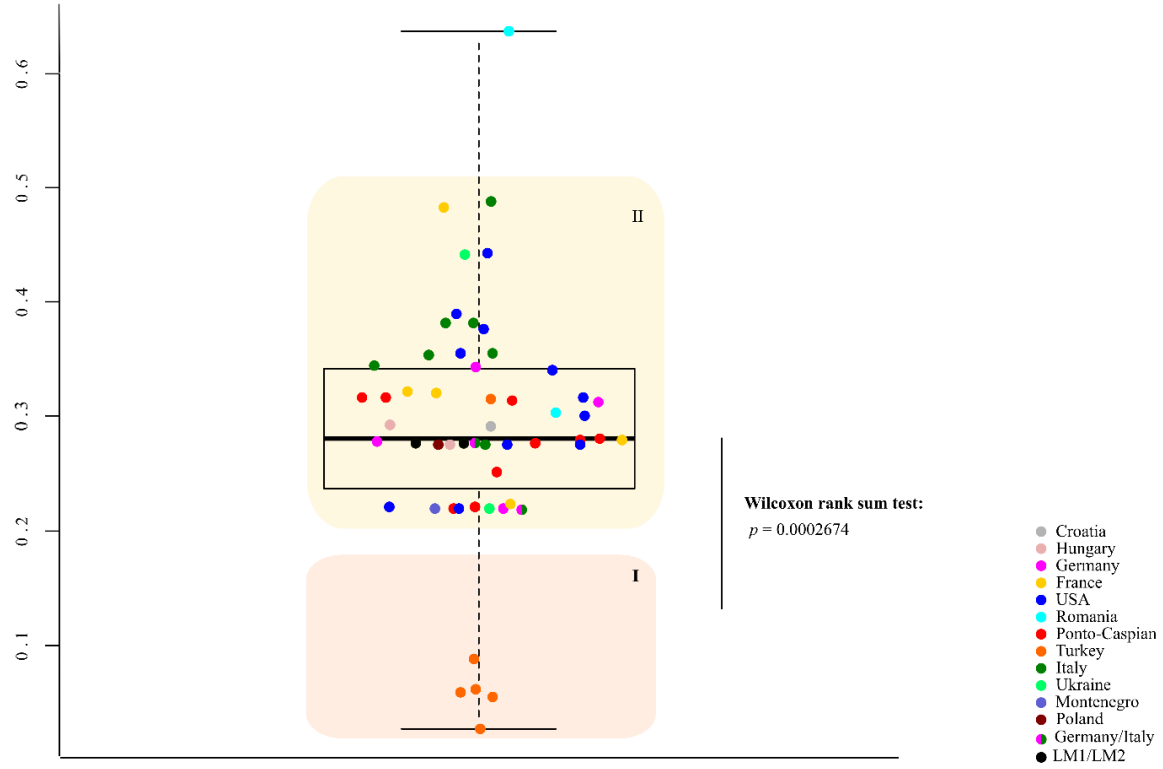


Figure 3.5. Linearized evolutionary distances (LED) of the *D. polymorpha* species.The species location are indicated by coloured circles.

3.5 Discussion

In this study, we investigated the demographic history of two invasive species from Northern Italy, *C. fluminea* (from Lakes Maggiore and Garda) and *D. polymorpha* (from Lake Maggiore), through a population genetic approach employing the mitochondrial COI gene. Our results demonstrated that both of these invasive species present similar genetic patterns, namely a low genetic diversity within the mitochondrial COI gene, which suggests that both *C. fluminea* and *D. polymorpha* derive from a reduced genetic pool. In addition, despite the different *Corbicula* spp. and *D. polymorpha* invasive repertoires, the observed polytomies in the inferred phylogenetic trees (Figures 3.3, 3.4 and 3.6) and the high sequence similarity suggest that these invasive species underwent a rapid dispersal in a short time period. It is noteworthy to mention that, in spite of the existing similarities between *C. fluminea* and *D. polymorpha* (*e.g.* natural traits, physiological tolerance, and high invasive behavior), *D. polymorpha* is confined to Europe and North America (Kinzelbach, 1992; Bij de Vaate *et al*., 2002; DAISE, 2008)

and has not spread to South American, African, Oceania and Asian continents, while *C. fluminea* is found across all continents (Figure 3.1). The widespread geographic distribution of *C. fluminea* may be attributed to its asexual reproduction mode, which seems to potentiate its invasive behavior (Pigneur *et al*., 2011, 2012, 2014).

Both database sequence comparison and phylogenetic inference of *C. fluminea* populations from both Maggiore and Garda Lakes (Northern Italy) reveal a single mtDNA COI haplotype, which has been previously detected in Europe (Renard *et al*., 2000), North and South America (Siripattrawan *et al*., 2000; Lee *et al*., 2005), and in the Asian native range (also denoted as haplotypes: I, form A and FW5, respectively) (Park & Kim, 2003). The FW5 haplotype is the most common haplotype in both the Asian region (Park & Kim 2003) and the extant invaded range (Siripattrawan *et al*., 2000; Lee *et al*., 2005; Marescaux *et al*., 2010; Pigneur *et al*., 2011), and it belongs to the FW5 invasive lineage. The FW5 lineage is generally characterized by *Corbicula* specimens with androgenic asexual reproduction mode (with biflagellate sperm), which is associated with an increased reproductive potential and a high invasive success (Hedtke *et al*., 2008; Hedtke & Hillis, 2010; Pigneur *et al*., 2011, 2012; 2014). In addition, the FW5 invasive lineage exhibits extremely low genetic diversity in the majority of the studied genetic markers (Lee *et al*., 2005; Pigneur *et al*., 2011; 2014; Gomes *et al*., 2016). The present study demonstrated that *C. fluminea* Italian populations present a low genetic variability in the mitochondrial COI marker, which is congruent with the characteristics of the FW5 androgenetic invasive lineage. Therefore, we suggest that the low genetic variability observed in these two populations from lakes Maggiore and Garda may be the result of an introduction of *C. fluminea* FW5 androgenetic asexual invasive lineage in the Po River in 1990s, which subsequently spread to other Italian ecosystems mediated through human activities, natural dispersion by birds, mammals and fish (Prezant & Chalermwat, 1984; McMahon, 2002; Karatayev *et al*., 2007).

Regarding *D. polymorpha*, a study conducted by Quaglia (2008) reported four haplotypes from the Italian Lake Garda (LG1 - LG4) and three other haplotypes (LC1 - LC3) from Lake Constance, Germany. In the present study, we only detected two of those haplotypes, the LM1 = LG1 as the dominant mitochondrial COI haplotype (represented by 98% of the total sampling) and the LM2 = LC3 haplotype, which is a rare haplotype. Futhermore, the mtDNA COI sequence comparison indicate that both LM1 and LM2 haplotypes have also been reported in other *D. polymorpha* populations across Europe and North America (Altschul *et al*., 1997; Benson *et al*., 2005). Previous studies hypothesize that the Italian *D. polymorpha* populations may have been

introduced mainly from Germany via human mediated activities (Giusti & Oppi 1972; Modena, 1994; Morpurgo & Thaler, 2002) and other European countries (Quaglia *et al*., 2008). In fact, it seems a plausible scenario, since both detected haplotypes LM1 and LM2 are present in German populations as well as other European countries. However, our phylogenetic analysis results cannot confirm with certainty the German introduction hypothesis but it does not excluded the European introductory event(s) because the Lakes Garda and Maggiore haplotypes clustered with other worldwide *D. polymorpha* populations, namely: Croatia, France, Germany, Hungary, Italy, Montenegro, Poland, Ponto-Caspian, Ukraine, Romania, Turkey and USA. Taken into account, the previous investigation (Quaglia et al. 2008) and the present study, we may hypothesize that the most parsimonious invasion scenario of *D. polymorpha* in Italy is as follows: *D. polymorpha* populations with a reduced genetic pool from Germany and other European countries where introduced in Lake Garda in the 1970s and posteriorly in Lake Maggiore in the 1990s. Furthermore, the gene pool of these repeated introductory events, most probably originated from the same population source and thus, explains the low genetic variability of the mtDNA COI observed in *D. polymorpha* populations from Lake Garda and Lake Maggiore.

Overall, we suggest that the both *C. fluminea* and *D. polymorpha* founded well-established populations in these studied ecosystems and successfully spread (through human mediated activities and/or natural vectors) to the majority of the Italian freshwater habitats giving rise to several genetically homogeneous populations.

3.6 Conclusions

The Italian *C. fluminea* and *D. polymorpha* populations from Northern Italy revealed a low genetic variability in the mitochondrial COI marker. Thus, we proposed that *C. fluminea* populations (Lakes Maggiore and Garda) originated from a reduced genetic pool belonging to the FW5 androgenetic invasive lineage, first introduced in the 1990s in Po River, and subsequently spread across other Italian rivers and lakes. A similar genetic invasive pattern was also observed in *D. polymorpha* populations (Lake Maggiore). We hypothesize that the Italian *D. polymorpha* populations originated from a reduced genetic pool from Germany and other European countries introduced in Lake Garda, in 1960s - 1970s and Lake Maggiore in the 1990s. Subsequently, once established these *D. polymorha* populations spread into other Italian freshwater

ecosystems. Thus, this mitochondrial COI genetic characterization study provided some insightful information regarding the introduction and spread of both *C. fluminea* and *D. polymorpha* populations in Italy.

3.7 Supplementary Materials

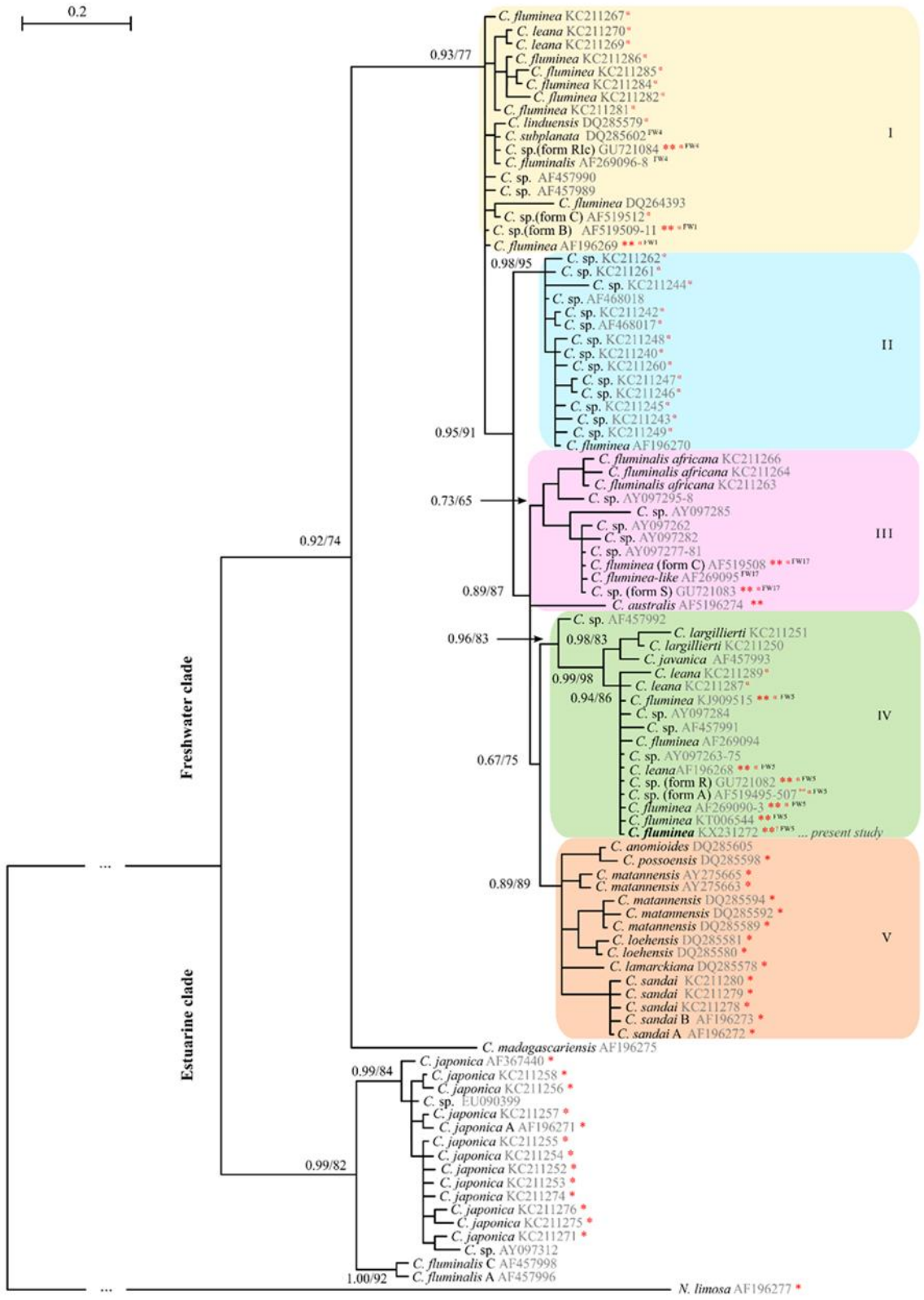


Figure 3.6. Bayesian phylogenetic tree of the mtDNA COI gene from *Corbicula* genus.Both Bayesian Inference posterior probabilities and Maximum Likelihood bootstrap values are indicated at the nodes.* Indicates the presence of monoflagellate sperm, ** indicates the presence of biflagellate sperm, α represents androgenetic lineages confirmed by cytological studies (Komaru et al. 1998; Glaubrecht et al. 2003; Ishibashi et al. 2003; Pigneur et al. 2014).

CHAPTER 4

Genetic diversity of the invasive bivalve *Ruditapes philippinarum* (Adam and Reeve, 1850) in Portugal

Gomes C, Mendes T, Borges R, Vasconcelos V, Guilhermino L and Antunes A (2017). **Genetic diversity of the invasive bivalve *Ruditapes philippinarum* (Adam and Reeve, 1850)** in Portugal. *Submitted to the Journal Aquatic Conservation: Marine and Freshwater Ecosystems*

4.1 Abstract

The bivalve *Ruditapes philippinarum* (Adams and Reeve, 1850) is a successful non-indigenous species (NIS) that has spread from the Indo-Pacific native range and was accidentally introduced into North America with imported Japanese oysters. Subsequently, it was intentionally introduced in Europe for commercial aquaculture. In Portugal, this invasive species was firstly reported in Ria Formosa (Algarve) in 1984. Since then it has naturalized many estuarine systems throughout the national territory, competing with the native populations of *Ruditapes decussatus*. Herein, we assessed the genetic diversity and the demographic history of *R. philippinarum* from Portugal employing three genetic markers – two mitochondrial COI, 16S rDNA and one nuclear 18S rDNA – in three estuaries namely, Aveiro, Óbidos and Sado. From the total haplotype variability detected within the COI (11 haplotypes) and the 16S rDNA (five haplotypes) genes, six of these haplotypes (COI3, COI5 COI6; 16S1, 16S4 and 16S5) are among the most common haplotypes found in the Atlantic and/or Adriatic populations. Thus, we hypothesized that the Portuguese *R. philippinarum* populations reflects the same dominant genetic variability as other European populations and the aforementioned haplotypes experienced a strong founder effect. However, a total of six rare haplotypes (COI8 - COI11 and 16S2 - 16S3) were reported for the first time in this Portuguese population study. These were most probably not detected previously due to sampling limitations and/or the occurrence of unreported introductions. Moreover, the phylogenetic inferences suggest that the Portuguese *R. philippinarum* populations genetic variability can be traced back to the Japanese and Chinese native genetic pool. However, as expected the studied Portuguese *R. philippinarum* populations showed a lower genetic diversity in comparison to the native range populations. Overall, our genetic characterization integrated with previous regional assessment evaluations, elucidated the demographic history of *R. philippinarum* in Portugal.

4.2 Introduction

The bivalve *Ruditapes philippinarum* (Adams and Reeve, 1850) is a successful non-indigenous species (NIS) that has spread from the Indo-Pacific native range (Ponurovsky & Yakovlev, 1992) into the North American and European coasts (Gosling, 2003). This species was first reported in North America west coast in 1936 and most probably was coupled with imported Japanese oysters (Quayle, 1964). Conversely, to other bivalve biological invasions, the *R. philippinarum* was intentional introduced in Europe as a food source for commercial aquaculture due to the human overexploitation of native European *Ruditapes decussatus* (Bald *et al*., 2009; Savini *et al*., 2010; FAO, 2017). Reports indicated that *R. philippinarum* was firstly introduced in France in 1972 for hatchery production and subsequently spread human mediated seed transfers into other European countries, namely Portugal, Spain, France, Italy, Ireland, United Kingdom (Gosling, 2003; FAO, 2017). Additionally, *R. philippinarum* seeds were also imported to other countries − French Polynesia, US Virgin Islands, Norway, Germany, Belgium, Tunisia, Morocco, and Israel (Figure 4.1) − for aquaculture trials (FAO, 2017).

Figure 4.1. Global distribution of *R. philippinarum*.Orange circles indicate the species distribution and the shaded green circle represents the Asian native range.

In Portugal, this invasive species was first reported in Ria Formosa (Algarve) and it has been proposed that it was introduced from Spain (Ruano & Sobral, 2000) most probably for aquaculture (Chainho *et al*., 2015). Since the 1980s until nowadays, *R. philippinarum* has naturalized many estuarine systems throughout the Portuguese

national territory (Gaspar, 2010; Chainho, 2014; Velez, *et al*., 2015a; Velez, *et al*., 2015b; Chainho *et al*., 2015). In fact, this species is one of the most abundant bivalves in Tejo, Ria de Aveiro and Sado estuaries (Chainho, 2014; Velez *et al*., 2015a).

The *R. philippinarum* physiological traits, such as, high fecundity, long larval phase, broad salinity and temperature tolerance has facilitated their dispersion and expansion (Breber, 2002). This contributes to the high sustainability of the aquaculture/fishing industry, since it is one of the most appreciated shellfish food and thus, presents a high economic value. Currently, the majority of the *R. philippinarum* production still comes from the Chinese native coastal region and is followed by European countries (Guo *et al*., 1999; Bald *et al*., 2009). Despite the economic importance, negative ecological impacts have also been reported in established *R. philippinarum* populations, the continuous dispersion of these populations has caused displacement of native species *R. decussatus* (Cohen & Carlton, 1995; Breber, 2002; Juanes *et al*., 2012; Bidegain & Juanes, 2013; Bendell, 2014). In addition, introgressive hybridization events have been detected between *R. decussatus* and *R. philippinarum* in the Indo-Pacific and European range (Kitada *et al*., 2013; Habtemariam *et al*., 2015). These introgressive hybridization events may lead to the native *R. decussatus* extinction by the replacement of hybrids through genetic mixing and thus, it is a major conservation concern (Allendorf *et al*., 2001).

As previously stated, *R. philippinarum* is a valuable resource for the fishing industries but it also has its negative impacts in the introduced ecosystems. The overexploitation of *R. philippinarum* in the native range and its expansion in the North American and European continents called the attention of the scientific community to further investigate this NIS and provide assistance with management support guidelines (Cigarría & Fernández, 2000; Bald *et al*., 2009; Choi *et al*., 2011). In fact, local and/or regional studies of *R. philippinarum* genetic diversity have also contributed to this species population management guidelines (Liu *et al*., 2006; Vargas *et al*., 2008; Chiesa *et al*., 2011; Mao *et al*., 2011; Mura *et al*., 2012; An *et al*., 2012; Xing *et al*., 2014; Chiesa *et al*. 2017). However, studies regarding the genetic diversity and population structure are still scarce in the native and non-native range (Astorga, 2014).

In the present study, we assessed the genetic diversity of *R. philippinarum* in Portugal employing two mitochondrial markers (COI and the 16S rDNA) and one nuclear

marker (18S rDNA). This genetic characterization assessment of the Portuguese *R. philippinarum* populations through the evaluation of the mitochondrial DNA COI will allow to make inferences regarding this species haplotype diversity, the genetic ancestry and effectively control existent quality of the Portuguese commercial stocks.

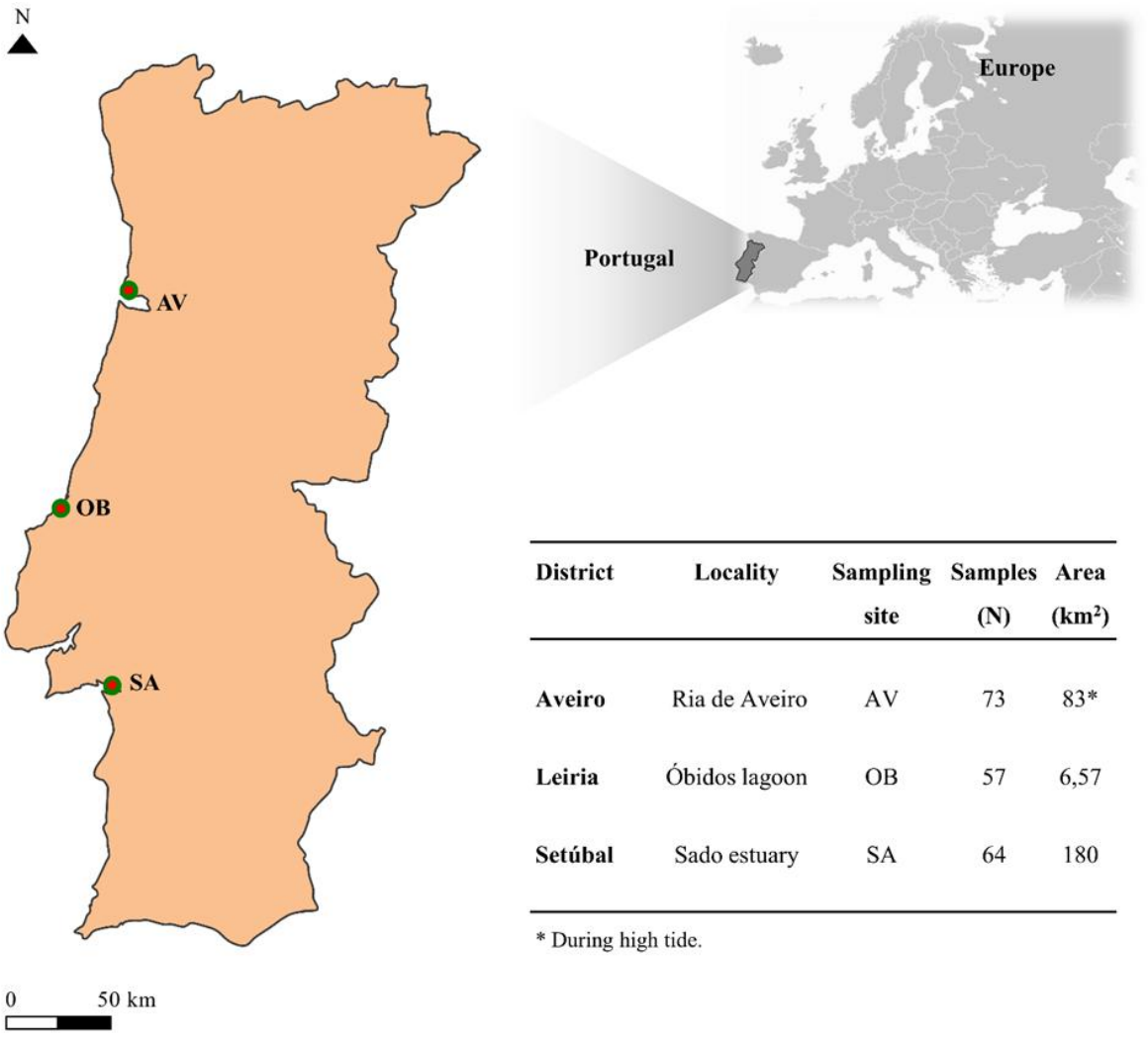
4.3 Materials and Methods

Ethics statement

The study did not involve any kind of endangered or protected species. No specific scientific research permits were required for the sample collection of these highly invasive invertebrates.

Study area and sample collection

A total of 194 *Ruditapes philippinarum* were randomly collected in three distinct Portuguese estuarine ecosystems namely, Aveiro (*N* = 73), Óbidos (*N* = 57) and Sado (*N* = 64) (Figure 4.2). The specimens were collected using a scoop net and/or by manually handpicked. Posteriorly, the clams were immediately transported to the laboratory and the soft body parts were individually isolated, preserved in absolute ethanol and stored at -80 ºC prior to DNA extraction.



District	Locality	Sampling site	Samples (N)	Area (km^2)
Aveiro	Ria de Aveiro	AV	73	83*
Leiria	Óbidos lagoon	OB	57	6,57
Setúbal	Sado estuary	SA	64	180

* During high tide.

Figure 4.2. *Ruditapes philippinarum* sampling areas in Portugal.Number of specimens collected per location and hydrological data, * tidal indication.

Genomic DNA extraction, amplification and sequencing

Genomic DNA was extracted from the foot *Ruditapes philippinarum* (*N* = 196) using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, California, USA) according to the manufactures instructions. All three markers (two mitochondrial, mtDNA COI, 16S rDNA and one nuclear 18S) were amplified in a total volume of 40 µl per reaction containing: 1x PCR buffer, 2.5 mM $MgCl_2$, 250 µM of each dNTP, 0.5 U of DNA Taq polymerase (Bioline, Luckenwalde, Germany), 10 pmol of a specific set of primers – LCO1490 and HC02198 for the mtDNA COI (Folmer *et al*., 1994), 16SAR and 16SAB for the 16S rDNA (Palumbi, 1996) and Myt18SF and Myt18SR for the 18S rDNA (Santaclara *et al*., 2006). The following PCR cycling conditions were used for the amplification of the mtDNA COI gene: 1 min at 94 ºC for initial denaturation, followed by

35 cycles of 1 min at 94 ºC, 30 s at 45 ºC, 1 min at 72 ºC and final extension of 10 min at 72 °C (Folmer *et al*., 1994) The 16S amplifications were performed with the following PCR cycling conditions: 5 min at 94 ºC for initial denaturation, followed by 35 cycles of 40 s at 94 ºC, 30 s at 52 ºC, 1 min at 72 ºC and final extension of 5 min at 72 ºC (Palumbi, 1996). The Myt18S rDNA gene reactions were performed with the following PCR cycling parameters: 5 min at 95 ºC for initial denaturation, followed by 35 cycles of 30 s at 95 ºC, 30 s at 50 ºC, 30 s at 72 ºC and final extension of 5 min at 72 °C (Santaclara *et al*., 2006). All PCR products were purified using Invitrogen PureLink™ Quick Gel Extraction & PCR Purification Combo Kit (Invitrogen, Carlsbad, California, USA) according to the manufacturer's instructions and the final PCR amplifications were confirmed by electrophoresis in a 1.5% w/v agarose gel stained with GelRed™ (Biotium Inc., Hayward, CA, USA). All final PCR amplifications followed direct sequencing (Macrogen, Amsterdam, Netherlands).

Genetic variation of the COI, 16S rDNA and 18S rDNA

A total of 582 DNA sequences were obtained for the three markers COI, 16S rDNA and 18S rDNA (194 sequences were yielded for each marker in three sampling sites namely, Aveiro, Óbidos and Sado). All sequence alignments were performed using default parameters of ClustalW in Geneious v.5.6.7 software (Kearse *et al*., 2012; Tamura *et al*., 2013; Larkin *et al*., 2007). The population genetic analysis was performed for each mitochondrial marker – COI, 16S – with the exception of 18S because of the detected low genetic variability. The DnaSP v. 5.10.01 software (Librado & Rozas 2009) was used to determine the number of haplotypes (h), haplotype diversity (Hd), nucleotide diversity (π), mean pairwise differences among sequences (k), and perform two neutrality tests – Tajima's D and Fu's F. The Network v. 4.611 software was employed to evaluate the phylogenetic relationships among the different populations through a median joining network (Bandelt *et al*., 1999). Analysis of Molecular Variance (AMOVA) based on the haplotypes frequencies were performed to determine differentiation among populations based on the F_{ST} values employing Arlequin v. 3.5 software (Excoffier & Lischer, 2010).

Phylogenetic analysis of the COI gene

A total of 125 *R. philippinarum* sequences were retrieved from the GenBank (Benson *et al*., 2005; Sayers *et al*., 2011) in order to perform phylogenetic analysis of the COI gene. The bivalve *R. decussatus* was used as outgroup. The jModelTest 2.1.1 was employed to estimate the best-fit nucleotide substitution model based on the corrected Akaike information criterion (with 95% confidence interval) (Guindon & Gascuel, 2003; Darriba *et al*., 2012). The phylogenetic tree construction of *R. philippinarum* employed the HKY + γ + I nucleotide substitution model. The phylogenetic inferences employed the Maximum Likelihood (ML) method in PhyML 3.0.1 (Guindon *et al*., 2010) using 1000 bootstraps replicates and Bayesian Inference (BI) in MrBayes 3.1.2, using 5000000 generations (Ronquist & Huelsenbeck, 2003). The BI trees were sampled every 1000th generation and 25% of the generated trees were excluded. The tree convergence was evaluated by the Potential Scale Reduction Factor (PSRF) and the Estimated Sample Size (ESS).

4.4 Results

Genetic variation – COI, 16S rDNA and 18S rDNA

COI gene

The 194 mtDNA COI *R. philippinarum* sequences analyzed yielded a total of 11 haplotypes (accession no. MG008471 - MG008481) denoted as COI1-COI11 (Figure 4.3). From the detected haplotypes, COI3 (40.2 %; *N* = 78), COI5 (20.1 %; *N* = 39), and COI6 (21.6 %; *N* = 42) present the highest frequencies and are represented in all the studied populations. The remaining haplotypes – COI1, COI2, COI4, and COI7 - COI11 – present lower frequencies (≤ 6.2 %) and were not detected in all the three studied populations. In comparison to previous studies, from the total 11 COI haplotypes, seven haplotypes − COI1 to COI7 − have been previously reported in the Atlantic and/or Adriatic populations (Chiesa *et al*., 2017). From those aforementioned haplotypes, four of them – COI3, COI4, COI6, COI7 – were common to the Atlantic and Adriatic populations namely, Portugal, France, Spain, UK and Italy. The haplotypes COI1, COI2 and COI5 were detected in the Atlantic region (Benson *et al*., 2005). The remaining haplotypes –COI8 to COI11– were detected here for the first time in our Portuguese *R.*

philippinarum populations but have been previously detected in the Asia native range, namely in Japan and South Korea (Benson *et al*., 2005).

Two distinct haplotype groups were observed in the mtDNA COI haplotype network (Figure 4.3), namely group I which englobes the mtDNA haplotypes COI4 - COI8, COI10 and COI11 and group II consisting of four mtDNA COI haplotypes COI1 - CO3 and COI9). Group I seems to present a COI haplotype distribution pattern mostly predominant of Aveiro and Óbidos (north/centre populations), whereas group II includes most of the centre/south populations namely, Óbidos and Sado.

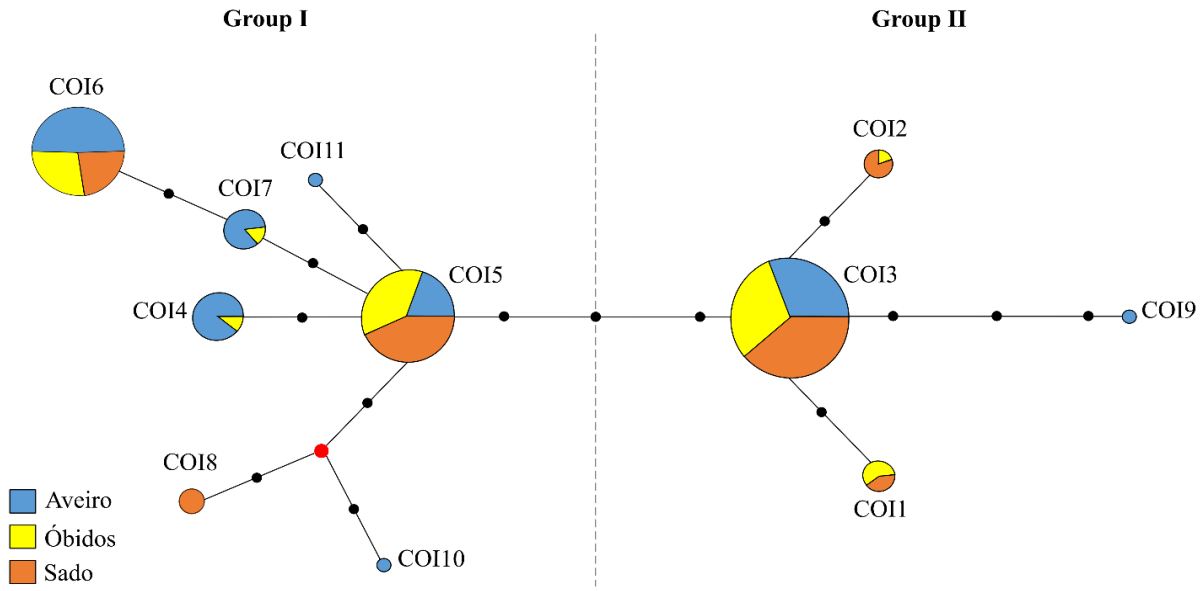


Figure 4.3. *Ruditapes philippinarum* median-joining haplotype network for COI gene.Circles represent COI detected haplotypes − COI1 to COI11 − and the circle size is proportional to their frequency in the sample of 194 sequences. The sectors within each circle represent the frequency of the haplotype in the three sampling locations. Number of mutation steps are indicated by the black dots and the red dot indicates a missing informative step.

The phylogenetic analysis of mtDNA COI gene clearly separates the *R. philippinarum* species (1/99 node support) from the *R. variegatus* (1/100, node support) (Figure 4.4). Within the *R. philippinarum* clade, two groups, denoted as group I, includes the haplotypes COI4 to COI8, COI10 and COI11, and group II encompasses COI1 to CO3 and COI9 haplotypes (with node support 51/62, respectively). This finding also corroborates the network analysis that suggests a mtDNA COI group distinction between the north/centre and the centre/southern Portuguese *R. philippinarum* populations. In addition, the Portuguese mtDNA COI haplotypes are mutually mixed with the *R. philippinarum* from Chinese and the Japanese native range.

Figure 4.4. *Ruditapes philippinarum* phylogentic tree of the mtDNA COI gene.Both Bayesian Inference posterior probabilities (BI) and Maximum Likelihood bootstrap values (ML) are indicated in the nodes, respectively.

16S DNA gene

The *R. philippinarum* 16S rDNA sequences (*N* = 194) yielded five haplotypes (accession no. MG008482 - MG008486), denoted as 16S1-16S5 (Figure 4.5). The haplotype 16S2 presents the highest frequency (88.1 %; n = 171) and was found in the all sampling points. The remaining haplotypes 16S1, 16S3-16S5 are present in much lower frequencies (≤ 4.1 %). No haplotype distribution pattern was evident within the 16S DNA gene.

The comparison with previous studies revealed that two mtDNA 16S haplotypes − 16S1 and 16S5 − have been previously reported as the most common haplotypes in the Atlantic and/or Adriatic populations, whereas 16S4 has been detected in Portugal (Chiesa *et al*., 2014). In addition, the present study also revealed two other haplotypes (16S2 - 16S3) which were identified for the first time in the Portuguese *R. philipinarum* populations but have also been reported in the European and native range populations, namely Spain and China, respectively (Benson *et al*., 2005).

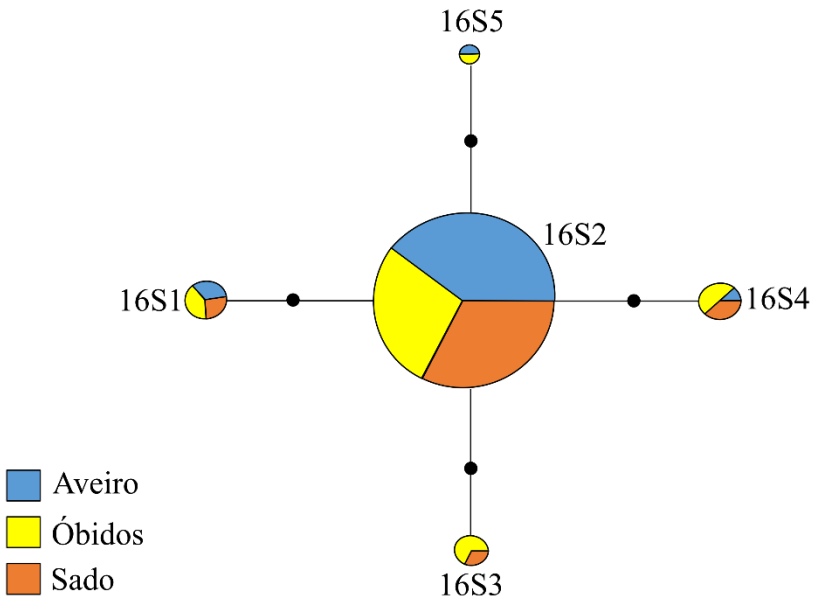


Figure 4.5. *Ruditapes philippinarum* median-joining haplotype network of the 16S rDNA gene.Circles represent the 16S rDNA detected haplotypes − 16S1 to 16S5 − and the circle size is proportional to the total haplotype frequency. The sectors within each circle represent the frequency of the haplotype in the three sampling locations. Number of mutation steps are indicated by the black dots.

18S rDNA gene

The 18S rDNA analyses reveal only one dominant haplotype (accession no. MG008487) from the total 194 *R. philippinarum* sequences. This haplotype has been reported previously in the Asian populations − Korea and China − and in Europe − Spain and the United Kingdom (Benson *et al*., 2005). No further analysis was performed in this dataset due to observed low genetic variability.

Nucleotide diversity and neutrality tests

The COI gene present the highest values for both haplotypes and nucleotide diversities (COI - Hd = 0.695 - 0.784 and π = 0.00351 – 0.0040, respectively) (Table 4.1). The 16S gene present the lowest haplotype and nucleotide diversities, which ranged from (Hd = 0.132 – 0.344 and from π = 0.00026 – 0.00071, respectively) in comparison the COI marker. Regarding the two neutrality tests only the 16S population of Aveiro presented a statistical significant value for both Tajimas´ D and the Fu´s F (D = -1,.489 and F = -3.746, respectively). All other values of Tajimas´ D and Fu´s Fs for the two analysis – COI, 16S – oscillated between positive and negative values but were statistically insignificant. The native range presented high haplotype and nucleotide diversities (Hd= 0.966 ± 0.013 and π =0.00510 ± 0.0004; data not shown) and highly significant Tajima´s value (D = -2.54623 $p < 0.001$; data not shown).

Table 4.1. Sampling locations, genetic diversity indices and values for neutrality tests for the COI and 16S rDNA.

Marker	Location	N	h	Hd	π (sd)	k	Tajima´s D	Fu´s F
COI	Aveiro	73	8	0.784 ± 0.024	0.0040 ± 0.00019	2.661	0.217	1.229
	Óbidos	57	7	0.712 ± 0.037	0.0036 ± 0.00021	2.395	1.013	1.174
	Sado	64	6	0.695 ± 0.041	0.0035 ± 0.00026	2.335	0.608	2.113
	Total	194	11	0.748 ± 0.020	0.00383 ± 0.0001	2.551	-0.016	0.552
16S	Aveiro	73	4	0.132 ± 0.053	0.00026 ± 0.0001	0.135	**-1.489**	**-3.746**
	Óbidos	57	5	0.344 ± 0.079	0.0007 ± 0.00018	0.371	-1.263	-2.746
	Sado	64	4	0.206 ± 0.067	0.00041 ± 0.0001	0.214	-1.298	**-2.670**
	Total	194	5	0.220 ± 0.039	0.00044 ± 0.0001	0.230	-1.187	**-3.293**

N = number of individuals, h = number of haplotypes, Hd = haplotype diversity, π = nucleotide diversity, k = mean number of pairwise differences among sequences and the significant values are shown in bold.

Analysis of Molecular Variance

The AMOVA based on the haplotype frequencies of the COI gene indicate that most of the genetic variation was detected within populations ≥ 95.6% (COI F_{ST} = 0.04360, p = 0.00880) and only ≥ 4% was assigned to comparisons among populations (Table 4.2). In addition, the pairwise comparisons of the COI also revealed a significant demarcation between Aveiro/Óbidos (COI F_{ST} = 0.04126, p = 0.02701) and Aveiro/Sado (COI F_{ST} = 0.0749, p = 0.00213). The 16S rDNA gene lacked of significant genetic variation for each surveyed population as well as within the all studied populations.

Table 4.2. Pairwise fixation index measured between sampling points.

		Aveiro	Óbidos	Sado
	Aveiro	–	**0.02701**	**0.00213**
COI	Óbidos	**0.04126**	–	0.53656
	Sado	**0.0749**	-0.00694	–
	Total	**0.0436**	**0.0088**	
16S	Aveiro	–	0.1639	0.46198
	Óbidos	0.00994	–	0.79078
	Sado	-0.0009	-0.01056	–
	Total	-0.00061	0.46725	

F_{ST} values are presented in the lower diagonal; p - values are presented in the upper diagonal; significant values are shown in bold.

4.5 Discussion

In this study, we assessed the genetic diversity and the demographic history of *R. philippinarum* from Portugal employing three genetic markers, two mitochondrial markers (COI, 16S rDNA) and one nuclear (18S rDNA). However, the COI gene revealed higher haplotype diversity than the 16S and 18S genes.

From the 11 COI haplotypes detected in the studied Portuguese populations, the COI3, COI5 and COI6 were the most dominant haplotypes which have also been reported as the most common haplotypes in European populations of *R. philippinarum* (Chiesa *et al*., 2017) and the native range namely, Japan and China (Mao *et al*., 2011;

Kitada *et al*., 2013). In addition, the haplotypes COI1, COI2, COI3, COI4, COI5, and COI6 are common haplotypes of the Atlantic and/or Adriatic populations namely, Portugal, France, Spain, UK and Italy (Chiesa *et al*., 2017). A previous COI genetic variability study of *R. philippinarum* in European populations, conducted by Chiesa (2017) revealed a total of 13 mtDNA COI haplotypes. In the present study, we also observed those Portuguese mtDNA COI haplotypes previously found in Italy, Spain and France (with the exception of the one haplotype). Interestingly, the remaining haplotypes mtDNA haplotypes COI8 - COI11 have detected in the Asia native range, mainly Japan but were also identified here for the first time in the Portuguese studied *R. philippinarum* populations (Benson *et al*., 2005).

The 16Sr DNA presented a total of five haplotypes (16S1-16S5) in the studied *R. philippinarum* populations from Portugal, from which two of those haplotypes − 16S1 and 16S5 − have been reported previously as the most common haplotypes in the Atlantic and/or Adriatic populations, whereas 16S4 has been detected in Portugal (Chiesa *et al*., 2014). In addition, the present study also revealed two other haplotypes (16S2 - 16S3) which were identified for the first time in the Portuguese *R. philipinarum* populations but have also been reported in other European and native range populations, namely Spain and China, respectively (Benson *et al*., 2005).

The 18S rDNA gene revealed a low genetic variability with only one main haplotype detected in this study. Therefore further analyses were not carried out due to the lack of genetic information regarding the 18S rDNA in the Portuguese *R. philippinarum* populations.

The neutrality test were not statistically significant in this COI dataset, only the Aveiro population present was statistically significant both 16S rDNA Tajimas´ D (D = -1,489) and the Fu´s F (F = -3,746) (Table 4.1). The COI AMOVA showed that most of the genetic variation detected in this study is mainly attributed to within populations (≥ 95.6%), whereas only ≥ 4% was assigned to comparisons among populations (Table 4.2). In addition, the pairwise comparisons of the COI showed a significant demarcation between the three sampling points the Aveiro with the Óbidos and Sado populations. This can also be observed in the haplotype diversity of the aforementioned markers, where the population of Aveiro presents higher diversity than Óbidos and Sado populations (Table 4.1).

Considering the overall haplotype diversity, the 16S gene presented a lower genetic diversity value (Hd = 0.220 ± 0.039) in comparison to the COI gene. However, the COI gene presented high haplotypes diversity values (Hd = 0.748 ± 0.020) but

slightly lower than the observed haplotype diversity in the native range (Hd = 0.966 ± 0.013). This finding was expected since previous studies have shown that the native range presents higher haplotypes diversities in comparison to the invaded regions of *R. philippinarum* (Mao *et al*., 2011; Kitada *et al*., 2013). Moreover, studies in the native range proposed that high haplotype diversity may be attributed to: *i)* large, stable, effective population sizes over time in species of the continental shelf (Stepien *et al*., 1999) and/or; *ii)* rapid population growth or sudden population expansion can also contribute to the genetic diversity conservation pattern of COI gene in *R. philippinarum*, due to the retention of the existent haplotype diversity and the slow rate of stochastic haplotypes loss by genetic drift (Rogers & Harpending, 1992; Avise, 1994). Even though, our COI results suggest that haplotype diversity of Portuguese *R. philippinarum* is slightly lower in comparison to the native range, this is most probably due to the founder effect and of the continuous human-mediated dissemination of this species that possess the same genetic pool.

The COI haplotype network indicated the existence of two distinct haplotypes groups (Figure 4.3), group I which consist of the north/centre populations and group II the centre/southern populations. In fact, the phylogenetic analysis (Figure. 4.4) also corroborates these findings, as two separate clusters were detected, group I and group II (51 and 62 node support, respectively). Although we cannot infer the *R. philippinarum* introductory route from our phylogenetic analysis, we may hypothesize that the origin source population of the Portuguese *R. philippinarum* populations ancestry may derived from both the Chinese and the Japanese genetic pools. In fact, this is a plausible scenario because it has been proposed that the North American and the European *R. philippinarum* populations derived from a Japanese ancestry, but possible unreported introductions from China may have occurred due to the high demand for industrial oyster aquaculture (Cordero *et al*., 2017). Actually, some Japanese and Chinese localities possess a mixture of the three *R. philippinarum* lineages (one Japanese and two Chinese) resultant of this species transfer between Japan, China and Korea for aquaculture activity (Mao *et al*., 2011).

The inference of the alien bivalve species invasion routes is invariably a complex task and the *R. philippinarum* is not an exception to this precedent. Nevertheless, taken into account this species establishment and genetic information assessment, it is possible to postulate its dispersal from the native range. The *R. philippinarum* was accidentally introduced in the North America continent in the 1930s from Japan (Cordero *et al*., 2017). The North American *R*. *philippinarum* species were then introduced in

Europe, primarily France and then the UK during 1972-1974. This same genetic pool was then introduced in other European countries – 1983 in Italy, 1990 in Spain, 1984 in Portugal (Chiesa et al. 2017) and 1987 in Norway (Palaz and Çolakoğlu 2014) – and other afar exploitation areas such as Turkey (Palaz and Çolakoğlu 2014). Interestingly, in the present study, we detected three dominant European COI haplotypes which derive from Japan and China (COI3 from Japan; COI5, COI6 from Japan and China). This is also concordant with our phylogenetic analysis, since the Japanese and the Chinese haplotypes did not cluster separately by region. Furthermore, other rare COI haplotypes (COI8 - COI11) were found in Portuguese *R. philippinarum* populations for the first time and have not been detected in the European range. Neverthless, the aforementioned haplotypes are from Japanese native range. Taken into account the aforementioned the *R. philippinarum* introductions/transferences and genetic information, we may hypothesize the following scenario: *i)* the *R. philippinarum* from North American and European populations share the same genetic pool composed from two main native origin sources namely, Japan and China; *ii)* the presence of the three dominant mtDNA COI haplotypes in the European populations suggest multiple introductions within non-native populations which explains the existence of a low genetic variability in comparison to the Asian region; and *iii)* the existence of rare haplotypes in Europe may be attributed to unreported *R. philippinarum* introductions and/or unintentional introductions coupled with oyster imports from the non-native and native range (Chiesa *et al*. 2017).

4.5 Conclusion

Overall, the Portuguese *R. philippinarum* populations genetic variability can be traced back to the Japanese and Chinese ancestry or genetic pool. Herein, the presence of the three dominant COI haplotypes – COI3, COI5 and COI6 – is most probably resultant of a strong founder effect which may attributed to the occurrence of multiple introductions from other European countries. Conversely, the presence of rare *R. philippinarum* haplotypes of the COI gene – COI8 to COI11 and 16S gene – 16S2 and16S3 – here detected for the first time in Portugal may be attributed to unreported introductions from non-native and the native range and/or were not previously found due to reduced sampling and the limited geographic study area. Nevertheless, this study has provided additional insightful genetic information that can be integrated with previous

regional assessment studies to further comprehend the genetic status *R. philippinarum* populations in Portugal. To conclude, it is important to assess the genetic characterization of *R. philippinarum* species for stock management and quality control purposes in order to prevent serious health problems for the final consumer, since this bivalve is widely used for human food consumption Lastly, the natural ecosystems should be monitored in order to avoid overexploitation and conservation of the native *R. decussatus*.

CHAPTER 5

General Discussion

5.1 General Discussion

This thesis general discussion focuses on the biologic aquatic invasions by invasive bivalves. The main sections (**chapters 2 - 4**) contribute to an enhanced comprehension of the biological invasions by non-indigenous species (NIS) that demonstrate to have both a high capacity of adaptation in freshwater and marine ecosystems. This is mainly attributed to the invasive species biological traits and the opportunities offered by the novel habitat (*e.g.* spatial availability, food resource and lack of other competitor bivalves and predators).

The overall scope of this research was to assess the molecular evolutionary genetics of invasive bivalves (**chapters 2 - 4**). In general, bivalves exhibit high plasticity, which can be attributed to their genetic makeup but also to the environmental selective pressures (*e.g.* biotic and abiotic factors). Therefore, in order to achieve an insightful scenario regarding bivalves bioinvasions, an integrative approach considering both ecological and molecular evidence were employed. Taken that into account, the aforementioned chapters included molecular experimental specimen data of three invasive bivalves – *C. fluminea*, *D. polymorpha* and *R. philippinarum* – the obtained raw data was analyzed through bioinformatic tools to assess genetic diversity and infer possible introduction route(s).

In this perspective, the screening of the genetic diversity of these invasive bivalves employed both mitochondrial DNA (mtDNA) and nuclear (nDNA) markers (with the exception of **chapter 3**, where only the mtDNA analysis was considered. In **chapter 2**, *C. fluminea* bivalve populations from Portugal were genetically evaluated employing two mtDNA markers (cytochrome c oxidase subunit I COI and cytochrome b (CYTb) and one nDNA marker (18S rDNA). In **chapter 3**, the *C. fluminea* and the *D. polymorpha* populations from Italy were surveyed using only the mtDNA COI marker, whereas two mitochondrial markers (mtDNA COI and the 16S rDNA) and one nuclear marker (18S rDNA) were employed for the genetic characterization of *R. philippinarum*, in **chapter 4**.

Currently, *C. fluminea* from Portugal present a low genetic variability within the COI gene, only one dominant haplotype was detected which corresponds to the European haplotype I (Renard *et al*., 2000), the North American haplotype form A (Siripattrawan *et al*., 2000) and the Asian FW5 haplotype (Park & Kim, 2003). Reports indicate that the *Corbicula fluminea* FW5 androgenetic invasive lineage possess

biflagellate sperm, thus being a diagnostic biological character indicative that the reproduction mode occurs asexually and through the androgenesis phenomenon (Hedtke *et al*., 2008; Pigneur *et al*., 2011). In this present study, the biflagellate sperm was detected in the Portuguese *C. fluminea* Douro population. Taken into consideration the presence of biflagellate sperm and the lack of genetic variability in all employed markers (COI, CYTb and 18S), we suggest that the Portuguese *C*. *fluminea* populations belong to the Asian FW5 androgenetic invasive asexual lineage (Konishi *et al*., 1998; Pigneur *et al*., 2011, 2012, 2014).

The majority of the previously performed research of *C. fluminea* populations from Italy is usually associated with ecological aspects, and no genetic screening had been performed in this invasive species until now. In **chapter 3** the genetic analysis of the Italian *C. fluminea* populations revealed the same pattern as the *C. fluminea* populations from Portugal, only one mitochondrial COI haplotype was detected, which corresponds to the European haplotype I (Renard *et al*., 2000), the North American haplotype form A (Siripattrawan *et al*., 2000) and the Asian FW5 haplotype (Park & Kim, 2003). Here, it was not possible to evaluate the sperm morphology due to specimen compound preservation. Regarding the *C. fluminea* morphometric analysis in Portugal (**chapter 2**), the principal component analysis (PCA) revealed two morphotypes, belonging to the northern population and the centre/southern populations, respectively. However, in the Lima River (northern population) the *C. fluminea* population was morphologically more alike the centre/southern populations. This analysis was also performed in the Italian *C. fluminea* populations, but no significant differences were detected (data not shown in **chapter 3**). In addition, both Portuguese and Italian *C. fluminea* populations exhibit a great shell plasticity (*personal observation*), which is mainly due to biotic and abiotic factors (Sousa *et al*., 2007) present in these invaded ecosystems (**chapter 2 and 3**).

Regarding the *Corbicula* genus (**chapter 2 and 3**), the native range exhibits higher haplotype diversity in comparison to the invaded regions. In fact, the Portuguese FW5 androgenetic invasive lineage of *C. fluminea* presents a low genetic variability and thus, we propose this may be attributed to the *C. fluminea* asexual reproductive mode which confers NIS a high invasive behavior. As aforementioned, it is presumed that the *C. fluminea* spread from the Asian native range into Portugal, Italy and other European regions, happened via the North American continent through ballast water discharge (Kinzelbach 1991). However, recent genetic studies are unable to confirm whether the

primary European introduction was via North and/or South America continent(s). Nevertheless, one cannot exclude that the *C. fluminea* FW5 lineage may have been introduced into the European continent directly from Asia also through discharge water ballast (Gomes *et al*., 2016). Overall, this research also corroborates previous genetic studies which clearly suggest that the *C. fluminea* FW5 androgenetic asexual invasive lineage presents low genetic variability and thus, might be attributed to the reproduction mode (Pigneur et al 2011, 2012, 2014). Neverthless, the genetic characterization, sperm morphology, morphometric analysis and distribution patterns (with exception of sperm morphology and PCA analysis in **chapter 3**) have provided new insightful information about this NIS genetic ecological data and genetic makeup which can be employed in additional future regional and/or worldwide studies. In **chapter 3** the Italian *D. polymorpha* genetic diversity was assessed employing the mtDNA COI and allowed to infer the introductory route/source(s) of this invasive species in two major Italian lakes – Lakes Maggiore and Garda. Since *D. polymorpha* populations from Lake Garda had been previously analyzed by Quaglia (2008), here the genetic characterization was only performed in Lake Maggiore and the results were posteriorly compared with those obtained previously in the other ecosystem by Quaglia (2008). The Lake Maggiore genetic assessment presented two mitochondrial COI haplotypes, one dominant denoted as LM1 (represented by 98% of the total sampling) and another rare haplotype the LM2, which was not previously detected in Lake Garda. The LM1 haplotype was also described as the dominant haplotype in Lake Garda. However, both of these haplotypes – LM1 and LM2 – were previously detected in *D. polymorpha* populations from Germany and sequence comparison analyses revealed that both of these haplotypes have also been reported across the European and North American continents (Altschul *et al*., 1997; Benson *et al*., 2005). It has been stated that Italian *D. polymorpha* populations derived from the German *D. polymorpha* populations, known as the "German introduction hypothesis" (Giusti & Oppi, 1972; Modena, 1994; Morpurgo & Thaler 2002). Considering the obtained results and comparison analysis of the studied *D. polymorpha* Italian populations, it seems that the most probable invasion scenario of this NIS resulted from the introduction of a reduced genetic pool from Germany but also from other European countries. Moreover, repeated or multiple introduction events must have occurred from the same genetic pool source and thus, explains the low observed genetic variability of the mtDNA COI in both evaluated Lakes Maggiore and Garda. Regarding the *C. fluminea* and the *D. polymorpha* distribution, it is noteworthy to mention that the *C. fluminea* population presents a worldwide distribution, whereas *D. polymorpha* seems

confined to the European and the North American continents (**chapters 2 and 3**) (Kinzelbach, 1992; Bij de Vaate *et al*., 2002; DAISE, 2008). This is probably due to different reproductive modes or strategies. Since *C. fluminea* presents an asexual reproductive mode it may confer a competitive advantage for increasing its potential invasive behavior (Pigneur *et al*., 2011, 2012; 2014) over the *D. polymorpha* sexual reproduction mode.

In summary **chapter 2 and 3**, propose that both *C. fluminea* and *D. polymorpha* invasion and dispersion is dependent of different dispersal vectors – human mediated activities and/or natural vectors – and their natural traits (*e.g.* physiological tolerance, and high invasive behavior) enables this NIS to successfully establish and dominate a novel ecosystem (McMahon, 2002; Karatayev *et al*., 2005, 2007; Sousa *et al*., 2006; Sousa *et al*., 2008; Pigneur *et al*., 2011). The low observed genetic variability of both *C. fluminea* and *D. polymorpha* is most probably due to the introduction and/or multiple repeated introduction events from the same gene pool and thus, instigates genetically homogeneous populations. Overall, these two chapters (**chapters 2 and 3**) corroborate previous *C. fluminea* and *D. polymorpha* researches but also contributed to fill in missing information in the assessment of these regional studies. Specifically, **chapter 2** presents a current and complete characterization status at the genetic and ecological levels, it also provides insights on possible introductory route(s) of Portuguese *C. fluminea* populations. Whereas, **chapter 3** also determines the existent genetic diversity and introduction pathways of *C. fluminea* populations in Italian freshwater ecosystem (Lakes Maggiore and Garda) which was not previously evaluated. In addition, **chapter 3**; assess the genetic variability analysis of *D. polymorpha* populations from Italy (Lake Magggiore) which was not previously genetically screened.

The historical demography of the intentionally introduced invasive marine bivalve *R. philippinarum* from Portugal was assessed in **chapter 4**. Here, a total of 11 mitochondrial COI haplotypes were detected, denoted as COI1-COI11. From these, the most frequent haplotypes in Europe and in the native Asian range are the COI3, COI5 and COI6 (Mao *et al*., 2011; Kitada *et al*., 2013; Chiesa *et al*., 2017)). In addition, some of these mitochondrial COI haplotypes – COI1 to COI6 – have also been reported in other European countries. However, the mitochondrial COI haplotypes – COI8-COI11– were detected in the Portuguese *R. philippinarum* populations for the first time and are also present in the Asian native range. Similarly, the mitochondrial 16S gene presented a total of five haplotypes – 16S1-16S5. From these mitochondrial 16S haplotypes, three

− 16S1, 16S4 and 16S5 − have been previously reported as common haplotypes in the Atlantic and/or Adriatic populations (Chiesa *et al*., 2014). Conversely, two haplotypes − 16S2 and 16S3 – were also detected in the Portuguese *R. philippinarum* populations for the first time but have been described in other European and native Asian countries (Chiesa *et al*., 2014). Regarding the 18S rDNA gene, a low genetic variability was detected, yielding only one dominant haplotype and thus, unabling the performance of further genetic analysis.

The mitochondrial COI network and the phylogenetic analysis exhibited similar patterns, indicating the possibility of two distinct mtDNA haplotype groups, group I belonging to the north/centre populations and group II the centre/southern populations. The neutrality tests indicate non-significant neutrality departures within the COI and the mitochondrial 16S dataset. In addition, for the COI gene most of the genetic variation detected (≥ 95.6%) was within the populations, while less than 4% was among populations. The mitochondrial COI gene presented a higher diversity in comparison to the 16S gene. Moreover, the mitochondrial COI dataset of the Portuguese *R. philippinarum* presents high haplotype diversity, but it is lower in comparison to the Asian native range populations. This is attributed to the strong founder effect and the continuous *R. philippinarum* seed transfers by humans from the same homogeneous genetic pool (Chiesa *et al*., 2014).

Considering the historical repertoire of *R. philippinarum*, unintentional introduction(s) into North America occurred from the Asian native range and posteriorly, intentional North American seeds were introduced in the European continent for aquaculture purposes (Utting & Spencer, 1992; Flassch &Leborgne, 1994; Albayrak *et al*., 2001; Jensen *et al*., 2004; Chiesa *et al*., 2017). Thus, it is plausible to assume that these populations derive from a similar genetic source which limits the haplotype diversity. However, the *R. philippinarum* introductory reports gathered with the obtained with genetic data from this study contributed with relevant information, namely: *i)* both populations of the *R. philippinarum* – from North America and Europe – share the same genetic pool and thus, explains the low genetic diversity in comparison to the *R. philippinarum* from the native regions; *ii)* the phylogenetic analysis corroborates that these *R. philippinarum* populations derived from Japan and China; *iii)* the presence of the three dominant COI haplotypes – COI3, COI5 and COI6 – in the European populations suggest that multiple introductions must have occurred from other European countries; and, *iii)* the existence of rare haplotypes detected in Portuguese *R.*

philippinarum populations may be due to unreported introduction events and/or even unintentional introductions due to bivalves food imports from the non-native and the native range and/or sampling bias (Cordero *et al*., 2017; Chiesa *et al*., 2017).

Overall, the thesis research discussed (**chapters 2 - 4**) depict the importance of the genetic characterization of these invasive bivalve species namely, *C. fluminea*, *D. polymorpha* and *R. philippinarum*. It contributes with fundamental and complementary information that can be combined with other biological information to achieve an enhanced understanding about the invasive behavior of these NIS, it also provides additional support for management conservational guidance to control current and future bivalve biological invasions and other potential invasive aquatic organisms.

CHAPTER 6

Conclusions and Future Perspectives

6.1 Concluding Remarks and Future perspectives

It is generally accepted that the biological invasion by bivalves usually provokes negative ecological and economic impacts due to their ability to cause major disruptions in the invaded ecosystem native fauna, structure and function. However, it is expected that biological invasions are likely to increase drastically due to globalization and it is extremely complicated to foresee biological invasions by non-indigenous species (NIS). Especially in the case of invasive bivalves which possess small dimensions at an early stage but have complete morphological and physiological functionalities. However, if some precautious actions are applied during the initial introduction phase, it would increase the chance to eradicate or at least control these invasive species. This would prevent and protect a potential endangered ecosystem from all the damage that bivalve invasive species may cause in its structure, function and biodiversity.

The human mediated activities are important vectors of the invasive bivalve dispersion – sport fishing, recreational activities, ballast water discharge, among others. A special attention should be ascribed to the discharge ballast water, since it is one of the most problematic issue that has contributed to the worldwide spread of these invasive bivalves. Therefore, it is prudent and essential to provide public awareness through the scientific community, as well as the politicians to implement harsher management guidelines for the preservation of these ecosystems biodiversity conservation.

In this scope, it is fundamental to closely monitor susceptible ecosystems to prevent invasive bivalve biological invasions and to control the existing ones. Moreover, monitoring vulnerable and/or invaded ecosystems ought to employ an “integrative study approach” englobing the taxonomy, ecology and molecular fields. This “integrative study approach” is essential for: *i)* comprehending the morphological and physiological features of the invasive organism; *ii)* determining the ecosystems function, structure and side effects caused by these invasive bivalves; and *iii)* providing insightful molecular knowledge by identifying and/or confirming with precision the invasive species in study, assessing the genetic diversity in order to obtain further inferences such as, possible introduction routes. Overall, this would certainly be an enormous achievement in order to obtain a more complete and realistic evaluation of the ecosystem before or after the occurrence of a biological invasion.

Lastly, over the last decade the invasive science field has significantly increased the attention of many scientific community members due to the invasive bivalves negative impacts in the ecosystem and associated economic costs. Even though much research has been performed in aquatic invasive species, it is never an easy task to perform complete biological researches because the majority of these studies focus mainly in regional and/or local case studies and thus, it is common to encounter missing data which limits future global worldwide studies. Herein, the scientific research community ought to make some efforts in performing and/or assist in national and international collaborations in order to provide more robust insightful global invasive science researches.

CHAPTER 7

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